Atypical Antipsychotic (AAP)-induced Metabolic Toxicity:

Study of Mechanisms, Biomarkers and Reversal Strategies

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Declaration

I hereby declare that the research reported in this thesis represents my own work carried out at the Wolfson centre for Personalised Medicine, Department of Molecular and Clinical Pharmacology, University of Liverpool, UK. This work has not been previously submitted to the University or any other institution in application for admission to a degree or other qualification except where otherwise indicated as help which is appropriately acknowledged.

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Abbreviations

≥	**Greater than or equal**
<	**Less than**
%	**Percentage**
ΔΔCT	**Delta delta cycle for threshold**
°C	**Degree Celsius**
µM	**Micromolar**
µg	**Microgram**
5-HT	**5-hydroxytryptamine**
AAPs	**Atypical Antipsychotics**
AMI	**Amisulpride**
ANOVA	**Analysis of variance**
ARI	**Aripiprazole**
au	**absorbance units**
BAT	**Brown adipose tissue**
BMI	**Body mass Index**
CA	**California**
CATIE	**Clinical Antipsychotic Trials of Intervention Effectiveness**
Cer	**Ceramide**
CerS	**Ceramide synthase**
CHD	**Coronary heart disease**
CI	**confidence Interval**
CLO	**Clozapine**
cm	**Centimetre**
CNS	**Central nervous system**
CVD	**Cardiovascular disease**
D2	**Dopamine receptor 2**

DEGS1	**Dihydroceramide desaturase**
DhCer	**Dihydroceramides**
DMEM	**Dulbecco's modified eagle's medium**
DMSO	**Dimethyl sulfoxide**
EDTA	**Ethylenediaminetetraacetic acid**
FBS	**Fetal bovine serum**
FDA	**Food and Drug Administration**
FFA	**Free fatty acids**
FGA	**First generation antipsychotics**
GWAS	**Genome-wide association study**
H	**Histaminic**
HALO	**Haloperidol**
HBSS	**Hank's balanced salt solution**
HEPES	**(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)**
hr	**Hour**
HRP	**Horseradish peroxidise**
IBMX	**3-isobutyl-1-methylxanthine**
IC	**Inhibitory concentrations**
IL-6	**Interleukin-6**
ILP	**Iloperidone**
IR	**Insulin Resistance**
IRS	**Insulin receptor substrate**
kg/m^2	**Kilogram per square metre**
LC	**Liquid chromatography**
LEP	**Leptin**
LPIN	**Lipin**
LPV	**Lopinavir**
MA	**Massachusetts**
MET	**Metformin**

mg	**Milligram**
MO	**Missouri**
µl	**Microlitre**
ml	**Millilitre**
mM	**Millimolar**
MS	**Metabolic syndrome**
MS	**Mass Spectrometry**
MTT	**(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)**
MVDA	**Multivariate data analysis**
ng	**Nanogram**
ns/NS	**Non significant**
OLA	**Olanzapine**
PBS	**Phosphate-buffered saline**
PCA	**Principal component analysis**
pg	**Picogram**
PLS-DA	**Partial least squares discriminant analysis**
PPAR-α	**Peroxisome proliferator–activated receptor alpha**
PPAR-γ	**Peroxisome proliferator–activated receptor gamma**
QTP	**Quetiapine**
RCT	**Randomised clinical trials**
RISP	**Risperidone**
RNA	**Ribonucleic Acid**
ROSI	**Rosiglitazone**
rpm	**Revolutions per minute**
RT-PCR	**Reverse transcription polymerase chain reaction**
SD	**Standard deviation**

SL	**Sphingolipids**
SPSS	**Statistical Package for Social Sciences**
SPTLC2	**Serine Palmitoyltransferase Long Chain Base Subunit 2**
T2DM	**Type 2 diabetes mellitus**
TNF-α	**Tumour necrosis factor-alpha**
TOPI	**Topiramate**
TX	**Texas**
VEH D	**Vehicle DMSO**
VEH E	**Vehicle Ethanol**
VEH M	**Vehicle Methanol**
WAT	**White adipose tissue**
ZIP	**Ziprasidone**

Abstract

Atypical antipsychotics are commonly prescribed for the treatment of psychiatric disorders such as schizophrenia. However, long term use of atypical antipsychotics lead to metabolic adverse effects such as weight gain, insulin resistance, and dyslipidemia, all of which are independent risk factors for cardiometabolic disease. Adipose tissue plays an important role in glucose and lipid homeostasis and has been linked to atypical antipsychotic-induced metabolic toxicity. The main aim of this thesis was to characterise the effect of atypical antipsychotics *in vitro* using murine 3T3-F442A preadipocyte cell lines and primary human adipocytes. Cells were incubated with clozapine (1-20μM), olanzapine (0.2-20μM) and aripiprazole (0.2-20μM) every 48 hours for 10 (3T3-F442A) or 13 (primary human adipocytes) days. Lipid accumulation was measured by Oil Red O assay. Protein and gene expression of peroxisome proliferator-activated receptor gamma and lipin 1 were measured by Western Blot and RT-PCR respectively. Adipokine secretion was measured by ELISA. Global lipidomic profiling of drug-treated adipocytes was undertaken using LC-MS and selected lipid species were validated by RT-PCR of enzymes that mediate the ceramide metabolism pathway. In differentiating 3T3-F442A cells clozapine (20μM:1.56 absorbance units±0.097; p=0.001) and olanzapine (20μM:1.57±0.14; p=0.07) but not aripiprazole showed an increase in lipid accumulation as compared to the vehicle. Clozapine but not olanzapine or aripiprazole upregulated the protein expression of peroxisome proliferator-activated receptor gamma (Mean fold change±SD; 307.34±26.30; p=0.0001) and lipin1 (213.46±26.43; p=0.02), and increased the secretion of adiponectin (736.77ng/ml±66.06; p=0.001; Vehicle: 323.26 ng/ml±53.07) and tumour necrosis factor alpha (45.24pg/ml±0.16; p=0.0001; Vehicle: 32.89pg/ml±0.34). In primary human adipocytes clozapine significantly increased lipid accumulation (1μM: 1.10±0.08; p=0.04) and leptin secretion (1μM: 1.06±0.10; p=0.04) but both clozapine and olanzapine led to a reduction in adiponectin secretion (Clozapine: 0.31±0.18; p=0.03, olanzapine: 0.39±0.21; p=0.01). Aripiprazole showed opposite effects to that of clozapine and olanzapine on lipid accumulation, gene expression and adipokine release. Drug uptake experiments showed that there was a 20-fold increase in clozapine uptake by differentiating primary human adipocytes (1293.4 pmol/million cells±170.56; p=0.006) in comparison to the murine adipocytes (64.25pmol/million cells±26.96). Co-incubation of clozapine with aripiprazole showed that aripiprazole significantly reversed clozapine-mediated reduction in secretion of adiponectin (1.23±0.09; p=0.04). Higher doses of both clozapine and olanzapine but not aripiprazole resulted in significant changes in the global lipidome profile of primary human adipocytes. Clozapine and olanzapine but not aripiprazole significantly decreased the ceramide 18 species and resulted in significant downregulation in the gene expression of ceramide biosynthesis enzymes, serine palmitoyltransferase long chain base subunit 2 (Clozapine: 0.60±0.13; p=0.003; Olanzapine: 0.62±0.16; p=0.007) and Delta-4-Desaturase Sphingolipid 1 (Clozapine: 0.67±0.18; p=0.01; Olanzapine: 0.83±0.11; p=0.02).

In conclusion, we have shown that atypical antipsychotics may cause metabolic toxicity by directly affecting adipocyte function and metabolism; however, this could be potentially reversed *in vitro* by various therapeutic strategies. Atypical antipsychotics also significantly alter the adipocyte lipidome which may contribute to metabolic adverse effects in schizophrenia patients. The mechanisms and pathways identified in this study now need to be validated in *in vivo* and clinical models which will aid the identification of toxicity biomarkers in atypical antipsychotic-treated individuals.

Chapter 1

General Introduction

1.1 Schizophrenia

Schizophrenia is a chronic, debilitating psychiatric disorder. It is estimated that approximately 0.5-1% of the world population is suffering from schizophrenia (Moustafa et al., 2016b). There is no specific age for schizophrenia to occur however the peak incidence of schizophrenia for men is between 10 and 25 years and 25 and 35 years for women (Rajji et al., 2009). Schizophrenia can occur at very late age, after 60 years, called as very-late-onset schizophrenia-like psychosis or in childhood after the age of 5 (Howard et al., 2000). The prevalence of schizophrenia is about 1 per 10,000 in children and 1-2 per 1000 in adolescents. Also in early adolescent, higher rates has been reported in young males compared to young females (Rajji et al., 2009).

1.2 Symptoms

Schizophrenia is characterised by (Weickert et al., 2000, Moustafa et al., 2016a);

Positive symptoms which include,

- hallucinations and
- delusions

Negative symptoms which include,

- lack of motivation,
- social withdrawal,
- disorganized speech and
- behaviour

Cognitive symptoms which includes impairment in,

- attention, memory and learning

According to Diagnostic and Statistical manual of Mental disorder (DSM-V) of the American Psychiatry Society and International Classification of Diseases (ICD-11) of the World Health Organization (WHO), the schizophrenia diagnostic criteria includes

two or more of the positive or negative symptoms for a significant portion of time during a one month period (Biedermann and Fleischhacker, 2016, Gaebel et al., 2013).

1.3 Risk Factors

Several risk factors have been identified which may lead to schizophrenia. Birth season is one of the factors identified with studies showing that schizophrenia may develop in individuals born in winter as compared to other seasons. This might be attributable to the infection which is more prevalent in winter especially in northern hemisphere (Messias et al., 2007). It was also found that individuals with antibodies to Toxoplasmosis *Gondi* have a higher prevalence to develop schizophrenia. Schizophrenia is also linked to herpes simplex virus or meningitis infections (Elsheikha and Zhu, 2016, Buka et al., 2008). Several studies and meta-analysis showed that the onset of schizophrenia is linked to prenatal and birth complications which includes prenatal exposure to infections, pregnancy complications, abnormal foetal growth and development or delivery complications (Cannon et al., 2002, Verdoux et al., 1997). Head injury has also been considered as a possible risk factor. Severe head injury has been associated with schizophrenia like symptoms (Dean and Murray, 2005). Drug abuse is also considered as possible risk factor for schizophrenia. Study conducted in Taiwan showed that large amount of methamphetamine use was associated with increased risk of schizophrenia (Chen et al., 2003).Cannabis use has also been found to increase the risk of schizophrenia (Messias et al., 2007).However there are studies which argued that cannabis does not linked to schizophrenia. More research is required to see cannabis association with schizophrenia (Sami and Bhattacharyya, 2018). Some studies also mentioned risk of schizophrenia with unemployment, low education, low socioeconomic status or being living alone. However more research is required to explore minority status association with schizophrenia (Dean and Murray, 2005). Numerous studies also mentioned that

individuals born in an urban environment develop schizophrenia much more than to those born in a remote area (Hosak and Hosakova, 2015, van Os et al., 2005, Torrey and Yolken, 1998).

1.3.1 Genetics

Family, twin and adoption studies have demonstrated that the susceptibility to schizophrenia is associated with genetics. Studies by Cardno and Lewis showed that the incidence rate for third degree relatives to be schizophrenic is approximately 2%. This incidence rate rose to 6% in second degree relatives and up to 17% in first degree relatives while the rate is 27% if both parents are affected (Cardno and Gottesman, 2000, Lewis and Lieberman, 2000). Also the incidence of schizophrenia is 17% in dizygotic twins and 50% in monozygotic twin. It has also been reported that there is 6-10 times more risk to develop schizophrenia in offspring in biological parents who already have schizophrenic disorder compared to adopted parents without disorder (Lewis and Lieberman, 2000). Linkage studies showed allele on chromosome 5 (5q11.2 to 5q13.3) might have linked to schizophrenia but it couldn't be replicated by others (Giegling et al., 2017). However, meta-analysis of genome-wide linkage studies of schizophrenia by Ng and colleagues suggested the evidence of linkage observed on chromosome 5q (142-168Mb) as well as 2q (103-134Mb). They also proposed that chromosomes regions 1, 2q, 3q, 4q, 5q, 8p and 10q might link to schizophrenia (Ng et al., 2009). Another meta-analysis, by Gatt and colleagues incorporating 97 schizophrenic variants separated the possible candidate genes linked with schizophrenia; these include; Catechol-O-methyltransferase (COMT), Dopamine receptor(DR) 2,3,4, D-Amino Acid Oxidase (DAOA), Gamma-aminobutyric acid type A receptor beta 2 (GABRB2), Methylenetetrahydrofolate reductase (MTHFR), 5-Hydroxytrptamine Receptor 2A (HTR2A), Interleukin 1 beta (IL1β) (Gatt et al., 2015).

Apart from isolated environment and genetic risk factors, studies are now focussing on gene-environment interactions. Evidence showed that concordance rate for illicit drug use was 22.3% for monozygotic twins and 14.5% for dizygotic twins (Oh and Petronis, 2008). This showed that individual who has an inherited risk for schizophrenia may have potential to use illicit drugs. Studies showed that polymorphism in catechol-O-methyltransferase gene modulated the influence of adolescent illicit drug use on developing schizophrenia (Caspi et al., 2005). Another study showed gene-environment interaction between pregnancy complications and polymorphism in genes, regulated by hypoxia, for increased risk for schizophrenia (Nicodemus et al., 2008). A combined genome-wide association study (GWAS) data extracted from International Schizophrenia Consortium (ISC) and European-American portion of the Molecular Genetics of Schizophrenia Consortium (MGSC) indicated a large number of genes which are associated with schizophrenia in interaction with environment. GWAS study found three novel candidate schizophrenia loci; NRGRN (neurogranin), TCF4 (transcription factor 4) and MHC (major histocompatibility complex) however, most of the schizophrenic GWAS showed association with MHC (International Schizophrenia et al., 2009, Stefansson et al., 2008, Giegling et al., 2017).However more research is required to explain the association between environmental risk factors and genes for schizophrenia.

1.4 Mortality

Schizophrenia, if not treated properly, can lead to adverse outcome which ultimately reduce the life span. Meta-analysis from 25 countries showed 2.5 times higher risk of death than the general population (Azad et al., 2016). A study by Laursen et al showed that overall mortality increase 2-3 times in schizophrenic patients compared to the general population (Laursen et al., 2013). There are both natural and unnatural causes of death in schizophrenic patients. Natural death arises as a result of cardiovascular

problems, respiratory diseases, poor compliance, cancer, unhealthy life style and antipsychotic effects. Unnatural death include suicide and accident which account for 40% of all death in schizophrenia (Ringen et al., 2014). Among the disease-related deaths, cardiovascular disease (CVD) is the leading cause of mortality. Several studies have shown that there are 90% higher deaths in schizophrenic patients compared to general population due to CVD (Harris and Barraclough, 1998, Ringen et al., 2014, Healy et al., 2012).

1.5 Risk factors of CVD in schizophrenic patients

Meta-analysis by Vancampfort and colleagues showed several risk factors may lead to CVD in schizophrenic patients. These include:- smoking, unhealthy eating pattern, sedentary behaviour, side effects of antipsychotics and low socio-economic status. According to National Cholesterol Education Program (NCEP), metabolic syndrome is one of the main risk factor for CVD (National Cholesterol Education Program Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2002). Stress involved in schizophrenia itself might lead to metabolic syndrome due to over activity of sympathetic nervous system (Scigliano and Ronchetti, 2013).Various studies have mentioned that metabolic syndrome is more prevalent among schizophrenic patients compared to general population (McEvoy et al., 2005, Weeke et al., 2014).

1.6 Pathophysiology

Interrupted connections in various brain regions including midbrain, nucleus accumbens, thalamus and prefrontal cortices might involve in pathophysiology of schizophrenia. Risk factors might have an effect on these neural circuits which ultimately lead to schizophrenia. Important neurotransmitters involved are dopamine, glutamate, serotonin and acetylcholine but dopamine and glutamate has been studied more (Lewis and Lieberman, 2000).

1.6.1 Dopamine hypothesis

Dopamine is produced in substantia nigra and ventral tegmental regions. It has connections to nigrostriatal, mesolimbic and mesocortical system and any disturbances in these connections cause abnormality in individual. Dopamine theory was suggested by Carlsson and Lindqvist, proposing that increased activity of dopamine transmission in brain leads to schizophrenic symptoms (Carlsson and Lindqvist, 1963). This theory was supported by other studies showing that dopamine releasing drug, for example amphetamine, as well as dopamine D2 receptor agonist, may increase the schizophrenia-like symptoms (Angrist et al., 1974, Creese et al., 1996, Seeman and Lee, 1975). Later it was found that drugs, for example chlorpromazine and haloperidol which block D2 receptor, improved the schizophrenic symptoms in patients of schizophrenia (Carpenter, 1996). However this theory was modified later proposing that hyper- and hypodopaminergic condition might occur in schizophrenic patients in different regions of the brain. It was further proposed that hyperactive dopamine transmission in subcortical areas trigger positive symptoms whereas negative symptoms and cognitive impairment may occur due to hypoactive dopamine transmission in cortical regions (da Silva Alves et al., 2008, Brisch et al., 2014, Svensson et al., 1995).

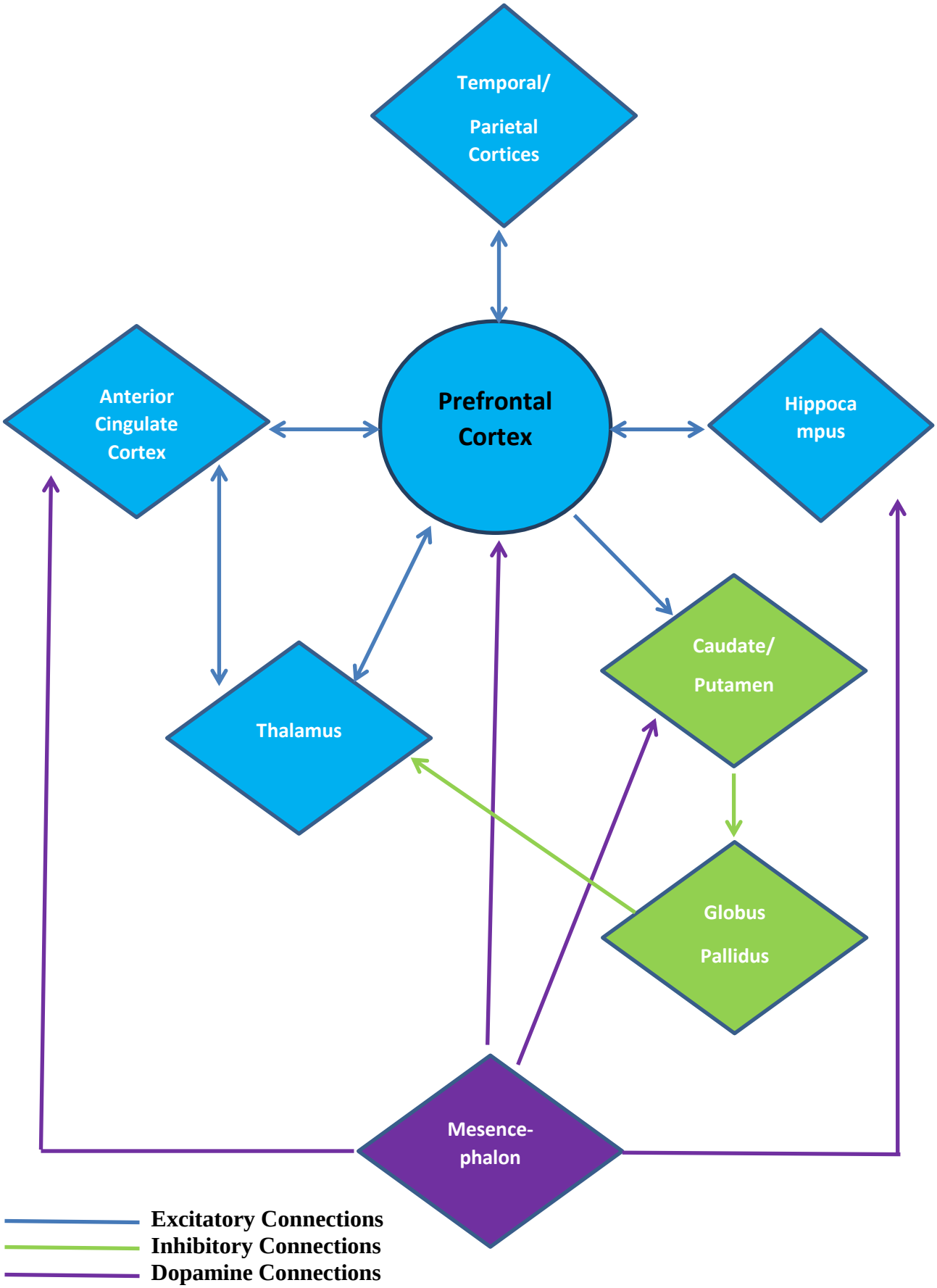


Figure 1.1 Affected brain regions in schizophrenia (modified from Lewis and Lieberman, 2000)

1.6.2 Glutamate Hypothesis

A study in healthy volunteers showed that glutamatergic N-methyl D aspartate (NMDA) receptor antagonist such as phencyclidine (PCP) or ketamine produced psychotic symptoms similar to what is seen in schizophrenia (Krystal et al., 1994). This was also confirmed in an experiment on mice which showed schizophrenia like symptoms on blockade of NMDA receptors (Mohn et al., 1999). This gave an idea that schizophrenic symptoms might involve dysregulation of NMDA receptor-mediated neurotransmission. This model is now currently used for aetiology and new treatment development of schizophrenia (Javitt, 2010).

1.6.3 GABA hypothesis

It was hypothesised that reduced gamma aminobutyric acid (GABA) neurons function could lead to psychotic symptoms (Roberts, 1972); however the result was inconsistent in other study (Deakin and Simpson, 1997). Recently Chang and colleagues suggested *Akt1* deficiency have effect on GABAergic interneurons and $GABA_AR$ expression, which can lead to cognitive functional impairment. This can lead the research towards development of GABA agonist in treatment of schizophrenia (Chang et al., 2016).

1.6.4 Serotonin Hypothesis

Serotonin or 5-hydroxytryptamine (5HT) has been proposed to have an effect in pathophysiology of neuropsychiatric disorders (Chang et al., 2016). However, results obtained from initial studies were disappointing although later studies identified the role of various 5HT receptors subtypes and their effect on other neurotransmitters on behaviour (Javitt, 2010). It was suggested that atypical antipsychotics improved schizophrenic symptoms by blocking $5HT_{2A}$ receptor (Veenstra-VanderWeele et al., 2000).

1.6.5 Cytokine Hypothesis

The cytokine hypothesis or model grabbed the attention of researchers for explaining the pathophysiology of schizophrenia. Several studies on adult rodents showed interactions between cytokines and dopaminergic signalling. Studies showed that giving repeated interleukin-6 (IL-6) along with amphetamine, produces symptoms similar to schizophrenia suggesting a role for cytokines in the pathophysiology of schizophrenia (Zalcman et al., 1998, Zalcman et al., 1999). The cytokine model is also in support of glutamate hypothesis which suggested that phencyclidine and ketamine induced schizophrenic symptoms. Connection between IL-6 and ketamine suggested that IL-6 could be mediator of ketamine which ultimately have devastating effect on paralbumain-positive (PV+) interneurons. These interneurons play role in cognition, disturbing them would lead to cognitive impairment (Kantrowitz and Javitt, 2010, Girgis et al., 2014, Lewis et al., 2005). Clinically various cytokines had been reported to be increased in schizophrenic patients which includes interleukins1, 2, 8, 6 and tumour necrosis factor-alpha (TNF-α) (Miljevic et al., 2013). It has also been reported that IL-6 levels increased in schizophrenic patients which then reduced significantly after treatment (Miller et al., 2011) but the data is not consistent as another study reported no effect of antipsychotics on plasma IL-6 levels. This inconsistent result suggest that effect on IL-6 may be due to illness itself instead of drug treatment (Himmerich et al., 2011).

1.7 Management

1.7.1 Non Pharmacological treatment

Various studies reported that psychosocial treatment is as important as pharmacological and patient can escape from the illness by using various ways (Rummel-Kluge and Kissling, 2008, Pharoah et al., 2010, Larsen et al., 2001).

Psychosocial intervention is suggested to be effective not only in improving patient's symptoms but also providing cost-effectiveness compared to standard treatment (Chien et al., 2013). There are 5 types of interventions which help the patients in their recovery. These include cognitive behavioural therapy (CBT) and cognitive remediation therapy (CRT), psychoeducation program, family intervention, social skills and Assertive community treatment (ACT) (Patterson et al., 2005, Chien et al., 2013). Among all 5 types, CBT and CRT are considered the most important in which the therapist usually evaluate and examine the thoughts and perceptions of the patients' symptoms and give a guided line to solve psychotic problems. It was suggested that CBT have a role in relieving positive symptoms but not negative symptoms (Pilling et al., 2002, Rathod and Turkington, 2005). However in terms of relapse there is difference in result. Gumley et al reported the significant effect of CBT (Gumley et al., 2003) while Durham et al showed a modest result in relapsing schizophrenic symptoms (Durham et al., 2003). Despite the differences in relapse, CBT still could be used as an adjunct to other psychosocial interventions. Cognition Remediation therapy is another intervention which include improved methodology related to neurocognition and social cognition, in order to improve the working memory, attention and psychomotor functions. McGurk and colleagues reported improvement in schizophrenic symptoms, memory and cognition abilities by using remediation technique during 1 year of follow up (McGurk et al., 2007). Several studies showed improvement in symptoms by going through psychoeducation programs, family-focussed intervention and social skills training (Pekkala and Merinder, 2002, Zapata Ospina et al., 2015, Weisman et al., 2005, Magliano et al., 2003). Another important non pharmacological intervention is ACT which is basically for refractory schizophrenia. This encompasses people who need immediate support, noncompliance with treatment and social breakdown. Clarke et al reported

improvement in symptoms and better life in patient used ACT technique (Clarke et al., 2000).

1.7.2 Pharmacological treatment

Antipsychotic drugs form the mainstay of treatment in schizophrenia patients. However due to neurological side effects of first generation antipsychotics, focus is now more on second generation or atypical antipsychotics (AAPs). Current guidelines recommended continuous therapy for one year after first psychotic episode and for 5 years or more in case of multiple episodes (Stip and Tourjman, 2010). Previously focus of pharmacotherapy was on synaptic modulation of dopamine system by acting on dopamine D2 receptors but studies on AAPs showed the role of other neurotransmitters like serotonin, norepinephrine, acetylcholine, histamine and glutamate (Lewis and Lieberman, 2000). These drugs have either low affinity for D2 and 5-HT2 receptors or might have effect through other dopamine receptors such as D 1, 2, 3, 4 or 5-HT1A, B, 2A, C, 3, 6, 7 . However AAPs also cause certain important side effects such as the metabolic adverse effects. To reduce these adverse effects, adjunctive drugs such as metformin, aripiprazole, topiramate and sibutramine are given along with AAPs. The details will be discussed in thesis chapter 4.

Others adjunctive drugs prescribed include benzodiazepines to control agitation; mood stabilisers to control mood; antidepressants to control depression; and, anti-cholinergics to reduce extrapyramidal symptoms (Lewis and Lieberman, 2000).

1.8 Antipsychotics under development or in clinical trials

1.8.1 N-methyl-D-aspartate receptor agonists

It was proposed that hypofunctioning of N-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission may produce schizophrenic symptoms. Whereas increased NMDA receptor activity will lead to have increased effect on glutamatergic circuit which ultimately improve the dopamine neurocircuit pathway resulted in

improvement in schizophrenic symptoms. Glycine and D-cycloserine are the most studied agents in relation to their effect on NMDA receptor. The cognitive and negative symptoms in schizophrenia trial (CONSIST) showed no improvement in schizophrenic symptoms by glycine and D-cycloserine (Buchanan et al., 2007). However the meta-analysis by Tsai et al showed improvements in schizophrenic symptoms by using glycine, D-serine and sarcosine in schizophrenic patients. Large-term clinical trials are required to determine their in-depth functional role in improvement of schizophrenic symptoms (Tsai and Lin, 2010).

1.8.2 Glycine Transporter 1 inhibitors

It was proposed that synaptic glycine levels were increased on blocking glycine transporter 1 which blocks glycine reuptake, and in turn improved NMDA neurotransmission. Sarcosine works by this mechanism. NMDA neurotransmission is useful for regulation of memory and cognition. A clinical trial of sarcosine showed improvement in schizophrenic symptoms however further studies are required to understand detailed mechanisms involved (Lane et al., 2010).

1.8.3 Kynurenic acid blocking agents

In brain, kynurenine pathways work by degradation of tryptophan and generate kynurenic acid (KYNA) metabolite which blocks glutamate and nicotine receptors. KYNA acts as an antagonist at the glycine site of NMDA receptor. KYNA levels were found to be increased in post-mortem brain of schizophrenic patients suggested KYNA involvement in psychotic symptoms. A Study on mice reported that targeted deletion of kynurenine aminotransferase II (enzyme that block the degradation of tryptophan) leads to low levels of KYNA and increased cognitive functions (Erhardt et al., 2009, Schwartz et al., 2012). Long-term clinical trials are required to get detailed role of KYNA blocking agents in treatment of schizophrenia.

1.8.4 Anti-inflammatory Agents

Anti-inflammatory agents can be helpful in improving symptoms of schizophrenia. It was reported in randomised clinical trials (RCT) that anti-inflammatory drug (celecoxib, a cyclooxygenase-2 inhibitor), which was used as an adjunctive drug in schizophrenic patients) showed partial improvement in positive and negative symptoms of schizophrenia (Muller et al., 2002, Muller et al., 2010, Akhondzadeh et al., 2007, Rapaport et al., 2005) while another trial which used aspirin, showed no improvement in symptoms (Laan et al., 2010). Various studies also reported improvement in schizophrenic symptoms by using drugs having anti-inflammatory effect such as minocycline, N-acetylcysteine, oestrogen, davunetide and corticosteroids along with antipsychotic drugs (Nitta et al., 2013, Sommer et al., 2014, Chaudhry et al., 2012). These studies broaden the way of developing new therapies targeting neuroimmune system.

1.9 Antipsychotic Drugs

Antipsychotic drugs are one of the most widely prescribed medications and mainly used for treatment of psychotic conditions like schizophrenia, mania and bipolar disorder (Newcomer, 2005).These drugs act by blocking D2 receptor in the central nervous system but they also affect other receptors such as D1, D4, 5HT, α-adrenergic, histaminic (H) receptors and muscarinic (M) receptors. Antipsychotics have been divided into two classes: the first generation antipsychotics are known as neuroleptic or typical antipsychotics and are potent dopaminergic D2 antagonists with extrapyramidal side effects; the second generation antipsychotics, also known as atypical antipsychotics (AAPs), show much less extrapyramidal side effects (Newcomer, 2007) .

1.9.1 History

The antipsychotic agents were originated from phenothiazine compounds which were initially used as antiseptics, anthelminthic and anti-malarials. In 1930, one of phenothiazine derivative, promethazine, was suggested to have antihistaminic and sedative properties. This was used by the anaesthetists during surgery to prolong and stabilise anaesthesia. Later on, another phenothiazine derivative, chlorpromazine, was synthesised by the French Pharmaceutical Company and it was found to reduce anxiety and induce mild sedation without causing loss of consciousness. In the early 50's, chlorpromazine was also found to be effective in improving symptoms in psychotic patients which led to its licensing in the USA in 1955 (Ban, 2007). In 1958, another antipsychotic, haloperidol (HALO) was synthesised and found to have neuroleptic activity (Ramachandraiah et al., 2009). In 1958, Carlsson and colleagues found that dopamine worked as an independent neurotransmitter in the brain. After few years, they suggested that both chlorpromazine and haloperidol increase the metabolism of noradrenaline and dopamine which provide the idea of involvement of dopamine in schizophrenic pathophysiology (Yeragani et al., 2010).

1.9.2 First generation (Typical) antipsychotics

The classification, chemical structure and receptor affinities of typical antipsychotics are given below depending on their chemical structure and potency (Katzung and Trevor, 2015).

Table 1.1 Classification of first generation (Typical) antipsychotics

(Modified from Katzung and Trevor, 2015)

Chemical Class	**Side Chain**	**Potency**	**Drug**
Phenothiazine	Aliphatic	Low/medium	Chlorpromazine Levomepromazine Promazine Trifluopromazine
	Piperidine	Low/medium	Mesoridazine Pericyazine Pipotiazine Thioridazine
	Piperazine	Medium/High	Perphenazine Fluphenazine Trifluoperazine
Non-Phenothiazine	Butyrophenones	High	Haloperidol Benperidol Droperidol
Thioxanthenes		Low/Medium	Clopenthixol Flupenthixol Thiothixene Zuclopenthixol
Dihydroindolones		Low/Medium	Molindone
Dibenzepines		Low/Medium	Clotiapine
Diphenylbutylpiperidines		High	Fluspirilene Pimozide

1.9.2.1 Mechanism of action

The first generation antipsychotics act by blocking D2 receptors in the CNS. They also act on 5HT2, H1, adrenergic and cholinergic receptors but D2 is the main receptor on which these drugs act. D2 receptors which are located in limbic and striatal areas of CNS are mostly responsible for antipsychotic efficacy and extrapyramidal side effects. Typical antipsychotics are found to be effective in first-episode schizophrenia. They also have improved effect on the positive symptoms but less effect on the negative symptoms as compared to AAPs. Typical antipsychotics also reduce the occurrence of relapse but do not provide much help in refractory schizophrenia as compared to AAPs (Joy et al., 2006, Lieberman et al., 2003b, Lieberman et al., 2003a, Thornley et al., 2003).

1.9.2.2 Adverse effects

1.9.2.2.1 Neurological

D2 receptors are important in locomotor functions especially through extrapyramidal motor system. Other functions related to D2 include learning, rewards, attention, memory, sleep and regulation of food intake. D2 receptor also control prolactin and aldosterone secretion and have role in vasodilation and gastrointestinal motility (Beaulieu and Gainetdinov, 2011). Due to the blockade of D2 receptor, it causes various neurological side effects; these include Parkinson's like symptoms such as bradykinesia, rigidity, tremor and appearance of mask-like face. Other effects include akathisia, where the patient become restless, agitated and distressed and show dystonic movement with muscular spasms limited to face, neck and back leading to abnormal posture (van Harten et al., 1999). After long-term treatment with typical antipsychotics, patients develop disfiguring and disabling effects involving irreversible involuntary movements, called as tardive dyskinesia. The symptoms comprise of continuous movement of tongue in and out of the mouth, movement of

fingers without any purpose and choreathetoid movement of entire limbs (Moore and Furberg, 2017). It is observed in about 3% of the patients who have used antipsychotics for long period of time. If not treated, it may lead to permanent disability (Chandra et al., 2017).

1.9.2.2.2 Endocrine effects

It has been recognised that typical antipsychotics increase the levels of prolactin. Infact, D2 receptor activation regulates the release of prolactin. On D2 antagonism, there is increased release of prolactin which causes hyperprolactinemia leading to gynaecomastia, galactorrhoea and erectile dysfunction (Haddad and Wieck, 2004). Several studies also showed that typical antipsychotics result in loss of bone mineral density (Liu-Seifert et al., 2004, Meaney et al., 2004, Meaney and O'Keane, 2007) and breast cancer (Harvey et al., 2008). No doubt, positive symptoms have been reduced in many patients with typical antipsychotics but these drugs have less effect on negative symptoms. It should also be noted that the D2 receptor occupancy by typical antipsychotics is more than 80% and that mediates these neurological adverse effects (Moore and Furberg, 2017).

1.9.3 Second generation antipsychotics (Atypical)

1.9.3.1 History

Clozapine (CLO) use started in 1960 and initially it was meant to be used as an antidepressant because of its chemical structure. However it was found later that it had neuroleptic properties as well (Hippius, 1989). It was reported that CLO was useful in the treatment of psychosis without causing extrapyramidal side effects and that attracted many researchers to do more research on this drug (Hippius, 1989). A later study, carried out in Finland, reported agranulocytosis as a severe adverse effect of

CLO (Crilly, 2007, Idanpaan-Heikkila et al., 1975); this resulted in the withdrawal of CLO from the market. However, CLO was studied extensively in the following years which resulted in the discovery of its effectiveness in resistant-schizophrenia and led to the re-entry of CLO back to the market in 1990 (Crilly, 2007). AAPs have been found to be cause less extrapyramidal side effects in comparison to typical antipsychotics. Extensive research on AAPs resulted in development of newer drugs with less adverse effects than typical antipsychotics and is discussed below.

1.9.3.2 Atypical antipsychotics drugs

The AAP drugs and their approval by the US Food and Drug Administration (FDA) are given below in the table;

Table1.2. Second generation (Atypical) antipsychotic drugs

(https://www.fda.gov/drugs/)(https://www.gov.uk/government/publications/antipsychotic-medicines-licensed-products-uses-and-side-effects/antipsychotic-medicines**)**

Name	**Abbreviation**	**Brand Name**	**FDA approval Year**	**Manufacturer**
Clozapine	CLO	Clozaril	1990	Novartis
Amisulpride	AMI	Solian	1990	Sanofi
Risperidone	RISP	Risperdal	1993	Janssen
Olanzapine	OLA	Zyprexa	1996	Eli Lilly
Quetiapine	QTP	Seroquel	1997	AstraZeneca
Ziprasidone	ZIP	Geodon	2001	Pfizer
Aripiprazole	ARI	Abilify	2002	Otsuka
Paliperidone	PLP	Travicta	2006	Janssen
Asenapine	ASP	Sycrest	2009	Lundbeck
Iloperidone	ILO	Fanaptum	2009	Vanda
Lurasidone	LSD	Latuda	2010	Sunovion
Brexipiprazole	BXP	Rexulti	2015	Lundbeck
Cariprazine	CPZ	Reagila	2015	Gedeon Richter

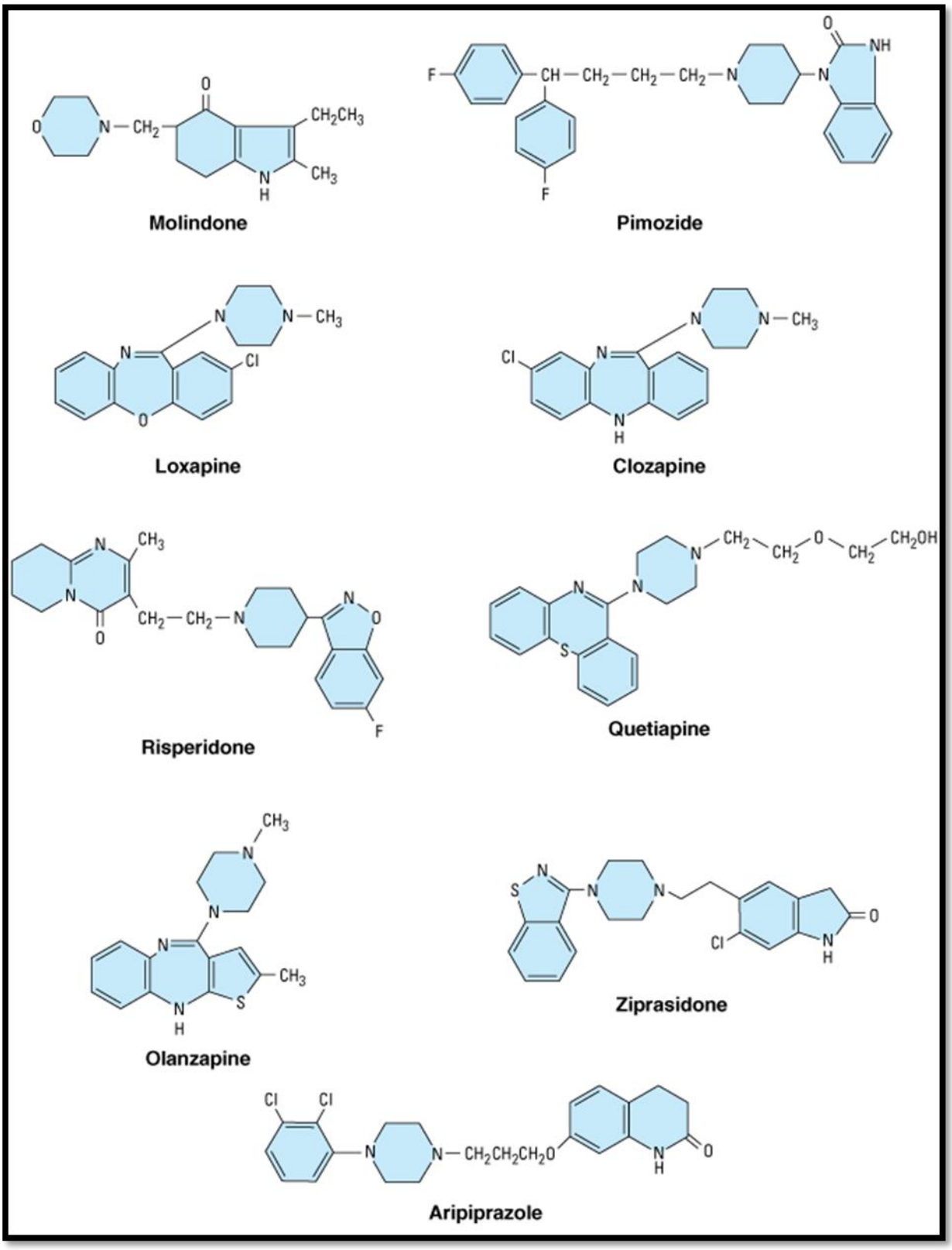


Figure 1.2 Chemical structures of Second Generation (Atypical) antipsychotics. (Adapted from Katzung and Trevor, 2015)

1.9.3.2.1 Clozapine

CLO is preferred over the first generation antipsychotics in the treatment of schizophrenia because of its better efficacy on treatment-refractory schizophrenia, reduced induction of extrapyramidal side effects, restoration of prolactin levels to its normal state and improvement in positive as well as negative schizophrenic symptoms (Mauri et al., 2014). In a study conducted by Lieberman et al (Lieberman et al., 2003a), better efficacy of CLO over chlorpromazine was found over a period of 52 weeks in a first episode schizophrenic patients. CLO has also been shown to be more effective for treatment-resistant schizophrenia than other AAPs in a meta-analysis carried out by Chakoe and colleagues (Chakos et al., 2001). CLO is better than HALO and RISP in reducing positive symptoms (Azorin et al., 2001, Volavka et al., 2002); it is more effective than switching to other AAPs (McEvoy et al., 2006) and significantly more effective than RISP, OLA, QUET and AMI in improving positive and negative symptoms (Lewis et al., 2006). Effectiveness of CLO was also shown in two of the largest randomized controlled clinical trials. In CUtLASS (Cost Utility of the Latest Antipsychotic drugs in Schizophrenia Study), a phase 2 clinical trial CLO was found significantly better than other AAPs in terms of efficacy and symptoms; it also improved the quality of life of schizophrenic patients. A second phase 2 trial, Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) also reported CLO to be better than other AAPs in the treatment of poorly responsive schizophrenia. However CLO did not show any evidence of its efficacy in first episode schizophrenia although it showed excellent effect in treatment-resistant schizophrenia (Naber and Lambert, 2009, Tandon et al., 2008). Studies also showed effectiveness of CLO in controlling aggressive behaviour in adults as well as in children having schizophrenia (Sarkar and Grover, 2013, Krakowski et al., 2006). Various studies have also shown the

effectiveness of CLO in bipolar disorder, mania (Arafat et al., 2016), reducing substance abuse and decreasing suicidal risks in schizophrenic patients (Iqbal et al., 2003).

1.9.3.2.1.1 Mechanism of action

Compared to other AAPs, CLO was found to have a greater effect in improving negative symptoms and cognitive deficits (Meltzer and McGurk, 1999). CLO has broader spectrum of receptor affinities; it not only act as an antagonist at several dopamine receptors (D2, D3, D4), but also on others receptors like serotonin 5HT2C, 5HT2A, adrenergic alpha (α) receptors, muscarinic (M) and histaminergic (H) receptors. CLO also act as a partial agonist at D1 and 5HT1A receptor which might play a role in its atypicality (Mauri et al., 2014). Through Positron emission tomography studies in schizophrenia, it was found that CLO showed 45-50% D2 receptor occupancy as compared to typical antipsychotics which occupied more than 80% .This might explain the atypicality and production of less extrapyramidal side effects by CLO compared to typical antipsychotics (Mauri et al., 2014, Wiesel et al., 1990). A study by Meltz and colleagues also suggested that CLO has greater D4 receptor occupancy than D2 and this might have some effect in its atypicality (Meltzer and Gudelsky, 1992). Serotonin receptor also plays an important role in the mechanism of action of CLO. It acts on 5-HT1A, 2A, 2C, 5-HT6, and 5-HT7 receptors. 5HT1A agonism and 5HT2A and 2C antagonism produce antipsychotic and improved cognitive effect. It has also been shown that increased dopamine release by CLO in rat prefrontal cortex might be due to 5HT1A receptor agonism (Meltzer and Massey, 2011, Meltzer, 1999).

1.9.3.2.2 Olanzapine

OLA is one of the common AAPs prescribed for schizophrenia. OLA, structurally and pharmacologically similar to CLO, is effective in treating schizophrenia, bipolar disorder as well as mania (Duggan et al., 2003). Compared to typical antipsychotics, OLA has proven to be a safer drug in terms of improvement in psychotic symptoms and adverse effects. It shows significantly reduced extrapyramidal symptoms along with decrease in prolactin levels. Chen et al reported improvement in positive symptoms of schizophrenic patients and decrease in prolactin levels when treated with OLA (Chen et al., 2009). Another study by Hartling and colleagues showed improvement in both positive and negative symptoms by OLA when compared with typical antipsychotics (Hartling et al., 2012). Johnsen and colleagues reported that OLA showed better treatment adherence and longer time to discontinuation compared to other AAPs showing advantage of OLA over other drugs. However it caused more metabolic adverse effects than other AAPs (Johnsen and Jorgensen, 2008). Komossa and colleagues gathered data regarding comparison of OLA with other AAPs; they reported both CLO and OLA as efficacious in improving symptoms. These contradict with CATIE and CUtLASS trials which showed superiority of CLO over other AAPs including OLA in terms of efficacy. However Komossa and colleagues argued that the dose might play role in the study; CATIE and CUtLASS used a dose between 500-600mg/day, whereas the maximum dose utilised in Komossa et al study was 400mg/day (Komossa et al., 2010a). The efficacy of OLA over QTP, RISP and ZIP in terms of mental state and symptoms has been reported; however there was limited data showing comparison of OLA with ARI (Komossa et al., 2010a).

1.9.3.2.2.1 Mechanism of action

OLA showed higher affinity to serotonergic receptors and binds to 5HT2A, 2C, 5HT3 and 5HT6. It also binds to D2, D1, M1, H1 and α1 and α2 receptors (Mauri et al., 2014). Another study conducted in rats reported the role of brain derived neurotrophic factor (BDNF) and tyrosine kinase receptors in schizophrenia. They showed the restoration of BDNF and tyrosine kinase receptors by OLA. However more research is required to explore the role of OLA in these receptors (Parikh et al., 2004).

1.9.3.2.3 Aripiprazole

ARI works by acting on D2, D3 and 5HT1A, 2A and 2B receptors. The unique point is that ARI act as a partial agonist at dopamine D2, D3 and serotonin 5HT1A receptors and possesses antagonistic activity at 5HT2A receptors. A study by Yokoi and colleagues observed no extrapyramidal side effects after giving maximum (30mg/day) dose of ARI in normal individuals (Yokoi et al., 2002). The exact mechanism of action is not known but it was suggested that ARI improves positive and negative symptoms of schizophrenia and has less tendency to cause extrapyramidal symptoms due to its partial agonist activity at D2 receptors. Because of its partial agonist activity at D2 and 5HT1A and antagonist activity at 5HT2A receptors, it has been called a dopamine-serotonin stabilizer (Mauri et al., 2014). In various clinical trials, ARI was compared with CLO, OLA, QTP, RISP and ZIP. It was reported that ARI showed similar efficacy but less adverse effects compared to other AAPs (Khanna et al., 2014). Various studies showed a range of ARI doses used, ranging from 10-20mg/day and it was found that doses 10-20mg/day showed greater improvement in positive and negative symptoms compared to placebo. This observation was further supported by other clinical trials such as Broad Effectiveness Trial with Aripiprazole (BETA) (Tandon et al., 2006) and the schizophrenia Trial of Aripiprazole (STAR). STAR was a 26 week randomized, multicentre trial that compared ARI with OLA, QTP and RISP

in terms of efficacy for the patients requiring antipsychotic medication switch; patients symptoms were found to improve after switching to ARI (Kerwin et al., 2007). ARI cause less adverse effect compared to other AAPs. In various trials ARI was administered in doses from 2 to 30mg/day for 6 weeks and akathisia was the only adverse effect observed (Stip and Tourjman, 2010). Less weight gain has also been reported with ARI by other studies (Stip and Tourjman, 2010, Newcomer, 2007, Kane et al., 2009). Because of it propensity to cause less adverse effects, ARI has become a more favourable drug to use as an adjunct or on its own.

1.9.3.2.4 Risperidone

RISP binds to serotonin 5HT2A, 7 and dopamine D2 receptors. It affinity to D3 and D4 is less than D2. It also binds to α1 and α2 adrenergic and H1 histaminergic receptors (Leysen et al., 1988). RISP is useful in schizophrenia and bipolar disorder and is effective both for positive and negative symptoms of schizophrenia (Rabinowitz and Davidson, 2001). According to Cochrane library database, RISP is not as effective as OLA but it is more effective than QTP and ZIP. RISP produced more extrapyramidal side effects and more prolactin when compared with CLO, OLA and ZIP and was also associated with more weight gain than AMI, ARI and ZIP but less than CLO, OLA and QTP (Komossa et al., 2011).

1.9.3.2.5 Quetiapine

QTP binds to serotonin 5HT2 and dopamine D2 receptors; however it has low affinity for D1 receptors. QTP has higher affinity to 5HT2 as compared to D2; this makes it more favourable to use in schizophrenia. It also bind to H1 and adrenergic α1 receptors and with lesser effect to α2, serotonin 5HT1A, 2A and 2C and muscarinic receptors (Mauri et al., 2014). Various studies have showed less efficacy of QTP compared to ARI (Citrome, 2012, Shoja Shafti and Kaviani, 2015). QTP produced less

extrapyramidal side effects than PLP, ZIP, ARI and OLA, but produced similar levels of weight gain to RISP and CLO (Asmal et al., 2013).

1.9.3.2.6 Amisulpride

AMI is dopamine D2 and D3 receptor antagonist but it shows no affinity at D1, D4 and D5. It also has less affinity for adrenergic, serotonergic and histaminergic receptors. AMI also produce less extrapyramidal side effects and it was suggested that it bind to D2 receptors for a short time and then dissociate quickly allowing dopamine transmission to follow its normal route (Seeman, 2002). On comparison with other AAPs, it was reported that AMI had the same efficacy as OLA and RIS but better efficacy than ZIP. AMI also induced less weight gain than RISP and OLA (Komossa et al., 2010b).

1.9.3.2.7 Ziprasidone

ZIP acts as a D2, 5HT2A, 2C and 1D receptor antagonist with greater affinity for the 5HT2A receptor (Schmidt et al., 2001). It also acts as a partial agonist at 5HT1A receptor. It does not block α1 adrenergic, M1 and H1 receptors (Mattei et al., 2011). Various clinical trials have reported that ZIP was less effective than AMI, OLA and RISP however it caused less weight gain than OLA, QTP and RISP. It also cause less cholesterol increase compared to OLA, QTP and RISP. However ZIP caused slightly more extrapyramidal side effects than OLA and resulted in more prolactin release than QTP (Komossa et al., 2009). No doubt ZIP show slightly increased extrapyramidal symptoms; however this is offset by the reduced impact of ZIP on body weight and effect on cholinergic, muscarinic and histaminergic receptors. Hence, this drug could be considered as metabolically friendly drug (Mattei et al., 2011). Another interesting point related to ZIP is its effectiveness in treatment-resistant schizophrenia is comparable to CLO. Kuwilsky et al reported that when CLO was combined with ZIP

or RISP, it resulted not only in the improvement in efficacy for long term but also improvement in CLO-associated adverse effects (Kuwilsky et al., 2010). Another trial, Monitoring Oral Ziprasidone as Rescue Therapy (MOZART), monitoring ZIP as an alternative to CLO in treatment-refractory schizophrenia reported that both CLO and ZIP have same efficacy. The authors suggested that ZIP was considered to be better compared to CLO as it caused less weight gain and less effect on cholesterol levels (Sacchetti et al., 2009).

Newer Atypical antipsychotics

Several newer antipsychotic drugs were approved by the US Food and Drug Administration (FDA) in recent times and the section below will discuss these agents in detail. In addition, clinical trials of various newly developed antipsychotic agents such as pomaglumetad methionil and bitopertin had been recently completed (Jaeschke et al., 2016).

1.9.3.2.8 Paliperidone

PLP is basically an active metabolite of RISP. It acts as an antagonist at D2 with predominant effect on serotonin 5HT2A receptors. It also has antagonistic effect on α1, α2 and H1 histaminergic receptors however it does not bind to muscarinic or β1 and β2-adrenergic receptors. Various studies have reported the effectiveness of PLP in improving schizophrenic symptoms (Fu et al., 2014, Zhang et al., 2015). It was also reported to cause moderate weight gain at therapeutic dose however at higher doses it caused extrapyramidal side effects (Bossie et al., 2017, Dlugosz and Nasrallah, 2007).

1.9.3.2.9 Asenapine

ASP has high affinity for serotonin receptors (5HT1A, 1B, 2A, 2B, 2C, 5HT5, 6, and 7), α adrenergic receptors, dopamine D1-D4 receptors and histaminergic receptors but

show less affinity for muscarinic receptors (Bobo, 2013, Peeters et al., 2011). A systematic review of ASP reported its effectiveness in controlling negative symptoms of schizophrenia and observed that ASP cause minimal adverse effects. However this review did not report any extrapyramidal side effects (Hay et al., 2015).

1.9.3.2.10 Iloperidone

ILP act as an antagonist on serotonin 5HT2A, dopamine D2, D3 and adrenergic α1 and α2 receptors. However it showed medium to low affinity for D3, 5HT1A, 2C, 5HT6, and histaminergic H1 receptors (Mauri et al., 2014). Various clinical trials have reported ILP to reduce positive and negative symptoms of schizophrenia and result in reduced weight gain and lesser extrapyramidal side effects (Scarff and Casey, 2011, Potkin et al., 2008, Cutler et al., 2008). It was also reported that ILP is as efficacious as HALO, RISP and ZIP in reducing schizophrenic symptoms and cause less adverse effects, favouring its use as first-line therapy (Tonin et al., 2016). A recent trial, RElapse PRevention Iloperidone EVidence Evaluation (REPRIEVE), conducted to evaluate the effectiveness of ILP for prevention of schizophrenic relapse reported that ILP was more effective than placebo in preventing relapse events in schizophrenic patients (Weiden et al., 2016).

1.9.3.2.11 Lurasidone

LSD act by antagonising dopamine D2 and serotonin 5HT2A and 5HT7 receptors. It also acts as partial agonist at 5HT1A and moderately acting at α receptor. It was reported that LSD was not as effective as CLO, OLA, AMI and RISP in terms of improvement of symptoms however it was effective than HALO, QTP, ARI, ZIP and ILP in the context of side effects (Jaeschke et al., 2016).

1.9.3.2.12 Brexipiprazole

BXP was approved in July 2015 by the FDA for its use in schizophrenia and as an adjunctive therapy to antidepressants. It acts as a partial agonist at dopamine D2 and serotonin 5HT1A receptors. In partial agonism it binds to receptor and activates it in condition where dopamine activity is decrease like in depression while it blocks D2 where high dopamine activity is there like schizophrenia. Animal data showed that BXP improved cognitive function by acting on the 5HT1A receptor. Various doses have been studied in clinical trials and it was reported in one trial that 2 and 4 mg of dose showed statistically significant improvement in PANSS score than placebo. Another trial reported 4 mg is efficacious than placebo. The common adverse effects related to BXP were reported to be akathisia and weight gain (Scarff, 2016a, Markovic et al., 2017). BXP has not been compared in detail with other AAPs. A review by Parikh et al reported BXP to cause less akathisia in comparison to Cariprazine (CRP); however BXP causes more weight gain than ARI, CPZ and ZIP (Parikh et al., 2017).

1.9.3.2.13 Cariprazine

CPZ was approved in September 2015 by the FDA. It acts as a partial agonist on D2 and D3 receptor and serotonin 5HT1A however it acts as antagonist at 5HT2A, 2B, 2C and 5HT7. There is minimal antagonism at histamine H1 receptors. Various trials on CPZ showed that in all doses studied (1.5, 3, 4.5 and 6) there was improvement in PANSS score. The most common adverse effects associated with CPZ were akathisia, extrapyramidal symptoms and headache. More clinical trials are required to characterise the CPZ profile (Scarff, 2016b).

1.9.3.2.14 Long acting Injectable (LAI) antipsychotics

Long acting injectable (LAI) are available for both typical antipsychotics such as HALO, Fluphenazine, Flupenthixol and Zuclopenthixol and AAPs such as OLA, ARI,

PLP and RISP. The purpose of LAI is to allow the physician to shift to long acting when non adherence occur with oral tablets and also to make the treatment easier. Meta-analysis showed effectiveness of LAI over oral antipsychotics in terms of relapse prevention (Leucht et al., 2011); however other studies showed no change in relapse prevention with both routes of administration (Fusar-Poli et al., 2013, Kishimoto et al., 2014). In terms of adverse effects, LAI proved to be worse than oral formulation. It was reported that LAI of OLA caused worsening of psychosis along with weight gain, extrapyramidal side effects and post injection syndrome. These adverse effects were also found to be associated with RISP and PLP. The most dangerous effect reported was leading to suicide and it was reported to be in patients taking RISP (Gentile, 2013). Further studies are required to explore more safety profiles of these LAI antipsychotics.

1.9.4 First generation vs. second generation antipsychotics

For the past few years, research focussed more on AAPs which have better therapeutic and less adverse effects compared to typical antipsychotics. Patient severities of schizophrenia are assessed by usually 2 scales, Brief Psychiatric Rating Scale (BPRS) and Positive And Negative Syndrome Scale (PANSS). Both provide overall psychopathology along with severity of positive and negative symptoms. A randomized controlled clinical trial reported effectiveness of AAPs over typical antipsychotics in terms of positive and negative symptoms. Depression, cognition and anxiety were also improved by use of AAPs (Tandon et al., 2008). Various other studies also reported effectiveness of AAPs over typical antipsychotics (Davis et al., 2003, Geddes et al., 2000). Meta-analysis of randomized controlled clinical trials by Leucht and colleagues comparing AAPs to HALO (typical antipsychotic) also showed effectiveness of all AAPs in improvement of schizophrenic symptoms over HALO (Leucht et al., 1999). Due to the greater efficacy and lower risk of motor adverse

effects, AAPs is becoming the gold standard for the treatment of schizophrenia compared to typical antipsychotics (Tandon and Fleischhacker, 2005).

1.10 Atypical antipsychotic-induced adverse effects

Compared to typical antipsychotics, AAPs show less extrapyramidal adverse effects; however, long term uses of AAPs are associated with potentially serious metabolic adverse effects. AAPs cause weight gain and associated metabolic disorders such as insulin resistance, impaired glucose tolerance, dyslipidemia and essential hypertension, all risk factors for the development of cardiometabolic disease (Nasrallah, 2008). The criterion for metabolic syndrome is given in the table below.

Table 1.3 Definition of metabolic syndrome (Sulistiowati and Sihombing, 2016, Huang, 2009)

	NCEP-ATP III (2005 revised)	**WHO (1998)**	**EGIR (1999)**	**1DF (2005)**
Criteria	Any three of the five criteria below	Insulin resistance (IGT, IFG,T2D or evidence of IR) or diabetes plus two of five criteria below	Hyperinsulinemia (plasma insulin >75 percentile) plus two of the four criteria below	Obesity plus two of the four criteria required
Obesity	Waist Circumference: > 40 inches (M), > 35 inches (F)	Waist/hip ratio: >0.90 (M), >0.85 (F) or BMI > 30kg/m2	Waist circumference: ≥94cm (M), ≥80cm (F)	Waist circumference ≥94cm (M), ≥80cm (F)
Hyperglycaemia	Fasting glucose ≥100mg/dl	Insulin resistance	Insulin resistance	Fasting glucose ≥100mg/dl
Dyslipidemia	TG ≥150mg/dl	TG ≥ 150mg/dl or HDL-C: <35mg/dl (M), <39mg/dl (F)	TG>177mg/dl or HDL-C<39mg/dl	TG ≥150mg/dl
Dyslipidemia (second separate criteria)	HDL cholesterol: <40mg/dl (M), <50mg/dl (F)			HDL cholesterol: <40mg/dl (M), <50mg/dl (F)
Hypertension	>130mmHg systolic or >85mmHg diastolic	≥ 140/90 mmHg	≥140/90mmHg	>130mmHg Systolic or > 85 mmHg diastolic
Others		Micro-albuminuria (urinary albumin excretion ≥20µg/min		

NCEP-ATP III (National Cholesterol Education Program-adult treatment plan III)
EGIR: European group for study of insulin resistance
IDF: International diabetes foundation
IGT: Impaired glucose tolerance; IFG: impaired fasting glucose: T2D: type 2 diabetes
IR: Insulin resistance, M: male; F: female, TG: Triglycerides
HDL-C: High density lipoprotein-cholesterol
≥ greater than or equal; < less than

Numerous studies including systematic reviews and meta-analysis showed that among AAPs, CLO and OLA appears to be associated with greater risk; QTP, RISP, ILP and PLP intermediate risk; while ARI, AMI and ZIP are associated with smaller risk of

metabolic syndrome (Table 1.4) (Smith et al., 2008, Papanastasiou, 2013, Bak et al., 2014, De Hert et al., 2007, Newcomer, 2005, Simon et al., 2009). The details of various characteristics of metabolic syndrome will be discussed in following sections.

Table 1.4 AAPs and risks for common metabolic abnormalities

Drug	Weight gain	Increased risk for diabetes	Lipid profile abnormalities
CLO	+ + +	+ + +	+ + +
OLA	+ + +	+ + +	+ + +
QTP	+ +	+ +	+ +
RISP	+ +	+	+ +
ILP	+ +	+	+
PLP	+ +	+	+
ASP	+	+	+
ARI	±	+	+
AMI	±	+	+
LSD	±	+	+
ZIP	±	+	+

+ + + **Strong effect**
+ + **Moderate effect**
+ **Less effect**
± **Mixed result**

1.10.1 AAP-induced weight gain

According to WHO, a body mass Index (BMI) (measured by weight in kilogram divided by square of height in meters) ≥25kg/m^2 is considered as overweight while ≥30kg/m^2 BM1 as obese. In the context of abdominal obesity, it is related to waist circumference >102 cm in men and >88cm in women. The prevalence of weight gain

ranges from 6% to 55%; these studies also suggested OLA as the main drug causing obesity (Nasrallah, 2008, Curtis et al., 2011, Tsan et al., 2012). Among AAPs, ZIP is considered to be the drug which causes the least weight gain while CLO and OLA are considered more likely to cause drug-induced weight gain. Various studies showed massive weight gain of 12.4 kg in CLO-treated schizophrenic patients while OLA showed 12 kg (Nasrallah, 2008, Nemeroff, 1997, Haddad and Wieck, 2004). A study by Haupt and colleagues showed that ZIP caused weight gain of only 1kg while RISP and QTP showed 2-3kg in first year of treatment (Haupt, 2006). A study by Nasrallah however, showed weight loss by ZIP (Nasrallah, 2008). ARI showed varying results of weight gain depending upon the BMI; in patients with a BMI of greater than 27kg/m^2, ARI showed weight loss instead of weight gain (Haddad, 2005). In other study in patients with a BMI less than 23kg/m^2, ARI showed a weight gain of 2.6kg (Nasrallah, 2008) (Figure 1.3).

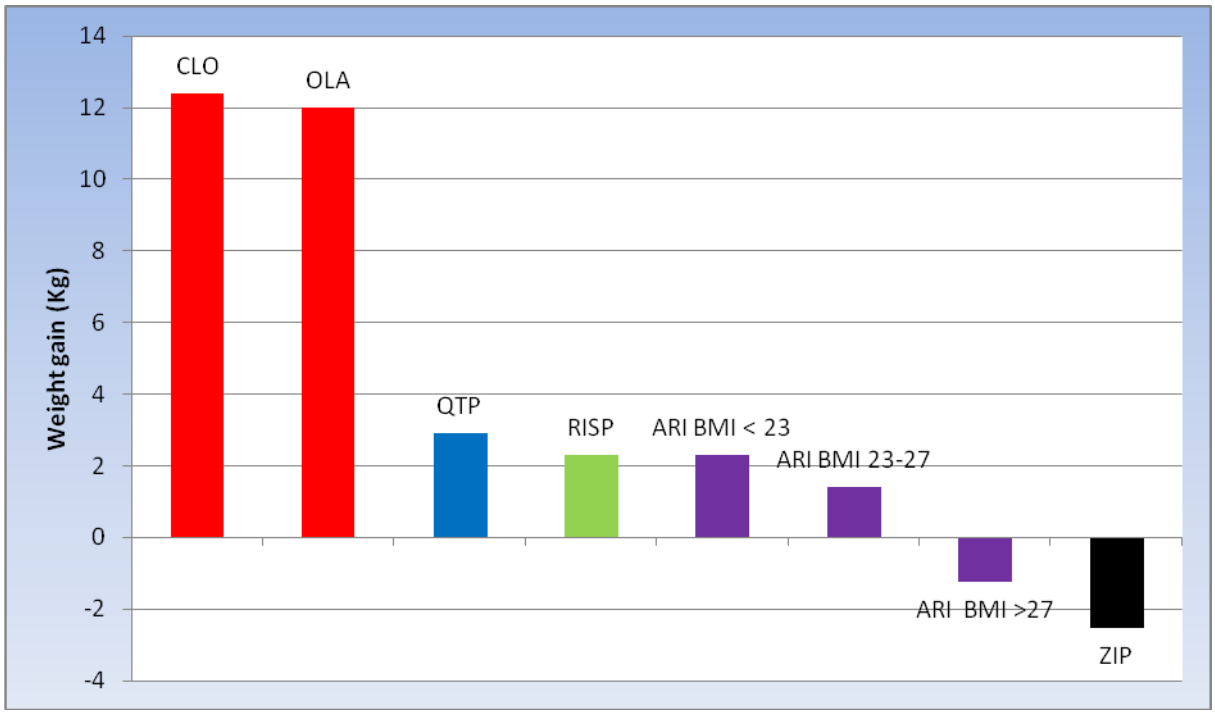


Figure 1.3 One year weight gain data in patients treated with AAPs (modified from Nasrallah, 2008)

The CATIE Schizophrenia trial which investigated the 10-year risk for coronary heart disease (CHD) in various AAP-treated patient groups identified that different AAPs confer varying levels of CHD risk with OLA being the most strongly associated with increased CHD risk (Swartz et al., 2008). CATIE trial was an 18-month, randomized controlled trial and showed comparison of 4 AAPs, OLA, QTP, RISP, ZIP and typical antipsychotic, perphenazine in 1493 schizophrenic patients. It showed that OLA, QTP, RISP and ZIP were associated with a weight gain/loss of +0.9kg/month, 0.23kg/month, 0.18kg/month and -0.14kg/month respectively. This showed that among the AAPs used, OLA caused the greatest weight gain with most adverse metabolic effects while ZIP caused the least adverse effects and weight gain (Swartz et al., 2008). Perphenazine no doubt was comparable to AAPs in efficacy but was discontinued due to high extrapyramidal adverse effects. However CLO, included in phase 2 of CATIE trial, was reported to be more effective drug for schizophrenic patients with a poor symptoms response to previous antipsychotic drugs although it was also associated with metabolic adverse effects (Swartz et al., 2008). European First-Episode Schizophrenia Trial (EUFEST), another 12-month clinical trial, conducted in 14 countries in 498 patients aged 18-40 years with first episode schizophrenia, compared HALO against AMI, OLA, QTP and ZIP. Patients who received OLA gained more weight (1.16kg/month) compared to QTP (0.88kg/month), AMI (0.81kg/month) and ZIP (0.4kg/month) (Kahn et al., 2008). CAFÉ (Comparison of Atypicals for First Episode) trial, a 52 week comparison of AAPs including OLA, QTP and RISP in schizophrenic patients, reported that OLA-treated patients showed more weight gain (average 1.76kg per month) compared to RISP (1.28kg per month) and QTP (1.29kg per month) (Patel et al., 2009). Several other studies also found CLO and OLA to produce the highest degree of metabolic adverse effects while QTP, RISP and AMI show intermediate effects; and ARI and ZIP are considered to cause the least

adverse effects (Kluge et al., 2009, Wang et al., 2013, Young et al., 2015, Newcomer, 2007).

1.10.2 AAP-induced hyperlipidaemia

The prevalence of hyperlipidaemia among AAP-treated patients was reported to be between 15-53% compared to general population (Mackin et al., 2007, Hagg et al., 2006, Young et al., 2015). Various studies showed that CLO and OLA cause high triglyceride and cholesterol levels in schizophrenic patients compared to RISP, AMI and ZIP (Ucok and Gaebel, 2008, Wu et al., 2006, Rettenbacher et al., 2006). Comprehensive literature review by Newcomer showed increase in cholesterol levels in schizophrenic patients after using CLO and OLA (Newcomer, 2005). CATIE trial also showed increased lipid levels after using OLA while ZIP produced less effect on lipids (McEvoy et al., 2006). Preclinical studies also showed increased lipid levels after administration of CLO and OLA (Boyda et al., 2010, Cooper et al., 2008, Minet-Ringuet et al., 2007). Increase in levels of triglycerides and cholesterols were reported in adult female rats after 48hr of single intraperitoneal injection of CLO and OLA (Jassim et al., 2012). Kalinichev and colleagues showed increased levels of free fatty acids in female rats after administration of OLA (Kalinichev et al., 2005). An epidemiological study carried out in Japan reported association of OLA with hyperlipidemia in AAP-treated patients compared to RISP, QTP, PLP, CLO and Zotepine while ARI was least associated with change in lipid levels (Takeuchi et al., 2015). Goncalves and colleagues showed that CLO and OLA caused more damage to lipid levels followed by RISP and QTP while ZIP and ARI were least associated with change in lipid levels (Goncalves et al., 2015).

1.10.3 AAP-induced hyperglycaemia

AAPs can increase the risk of hyperglycaemia ultimately leading to diabetes mellitus or diabetic ketoacidosis. The prevalence of high glucose in schizophrenic patients receiving AAPs is reported to be 24.6% (Ko et al., 2013, Young et al., 2015). A meta-

analysis by Jin and colleagues showed increased glucose levels developed within 1-3 months of use of AAPs (Jin et al., 2002); this was supported by Balf et al who also found hyperglycaemia to develop within 3 months use of CLO, OLA, QTP and RISP (Balf et al., 2008). Various studies have also associated CLO and OLA with increase in glucose levels, glucose intolerance and insulin resistance compared to other AAPs (Newcomer et al., 2002, Ober et al., 1999, Goncalves et al., 2015). CLO and OLA were also associated with increased risk of diabetic ketoacidosis (DKA) and diabetes (Newcomer, 2005). Though DKA usually develop in type 1 diabetes, reports have shown incidence of DKA in patients receiving AAPs especially CLO and OLA (Balf et al., 2008). FDA adverse event reporting system database (FAERS) mentioned OLA as the most notorious drug causing hyperglycaemia among all AAPs (Kato et al., 2015). Meta-analysis by Rummer-Kluge also showed strong association of CLO and OLA in causing hyperglycaemia compared to various other AAPs (Rummel-Kluge et al., 2010). Interestingly, various studies have also reported reversal of glucose levels to nearly normal level by switching to RISP, ARI or ZIP. In these studies, patients with schizophrenia on CLO and OLA showing metabolic adverse effects were included. Clinical diagnosis was evaluated using CGI-S and PANSS scale. It was reported that switching to RISP, ARI or ZIP for 4-6 months not only decrease body weight but also decreased glucose and cholesterol levels to nearly normal levels (Montes et al., 2007, Wani et al., 2015, Weiden et al., 2003, Bernardo et al., 2011).

1.10.4 AAP-induced cardiovascular disease

The prevalence of CVD in AAP-treated schizophrenic patients is 1.5-2 times higher than general population (Kelly et al., 2010). It was reported that AAPs contribute to the development of CVD and stroke by increasing the risks such as hyperglycaemia, weight gain and hyperlipidaemia. Daumat and colleagues assessed the antipsychotic drugs effects used in CATIE on participants' 10-year coronary heart disease (CHD) risk. It was found that risk for CHD differs significantly between antipsychotic agents,

with OLA showing the greatest elevation in CHD risk (Daumit et al., 2008). The prevalence of hypertension, an independent risk factor for CVD, was reported to be 16-49% in schizophrenic patients treated with AAPs (Ko et al., 2013, Young et al., 2015) with CLO the most associated (Henderson et al., 2004, Gupta and Rajaprabhakaran, 1994, George and Winther, 1996). Study by Woo and colleagues showed CLO but not OLA cause hypertension (Woo et al., 2009). The CATIE trial also did not show any change in blood pressure in all of the AAPs used in schizophrenic patients (Boden et al., 2013). However, Jerel et al reported ZIP to be involved in hypertension (Jerrell et al., 2010). Prolongation of QTc interval is also an important adverse effect. It may leads to *torsade de pointes*; a ventricular arrhythmia which if not treated leads to ventricular fibrillation and then cardiac arrest. In CATIE trial there was no effect on QTc interval among OLA, RISP, QTP and ZIP. However in one clinical trial comparing sertindole and ZIP, QTc was reported to be increased by sertindole (Glassman and Bigger, 2001). This contradicts with another trial where ZIP was reported to prolong the QTc interval in schizophrenic patients (Wilton et al., 2001), an observation supported by Khasawneh and Shankar (Khasawneh and Shankar, 2014). Myocarditis, a rare adverse effect, was reported to develop in CLO-treated schizophrenic patients (Khasawneh and Shankar, 2014). The data regarding association of AAPs with myocardial infarction (MI) have been inconsistent and conflicting. Some studies reported an increased risk of MI associated with AAPs (Huang et al., 2017, Enger et al., 2004) whereas others found no association (Jerrell and McIntyre, 2007, Nakagawa et al., 2006). Arteriosclerosis is also considered as an important risk factor leading to CVD. Hypertension and other diseases like diabetes increase the stiffness of arteries. A study by Findikli and colleagues reported increased arterial stiffness in AAP-treated schizophrenic patients (Findikli et al., 2016). Risk factor such as remnant-like lipoprotein particles cholesterol (RLP-C) also play

important role in development of CVD. A study by Nagamine, comparing effects of RISP and OLA on RLP-C in schizophrenic patients showed more RLP-C levels by OLA compared to RISP. It is suggested that RLP-C may take up by macrophages in arterial wall where it form foam cells leading to formation of atherosclerotic plaques (Nagamine, 2008). It has already been demonstrated that RLP-C promote arteriosclerosis through formation of atherosclerosis plaque due to increased uptake by macrophages, impaired vascular endothelial cell function, increased platelet aggregation and enhanced monocyte adhesion to vessel endothelium (Tanaka, 2004). It was proposed that atherosclerosis in coronary artery might lead to arteriosclerosis leading to CHD (Nagamine, 2008). Because of some confusing results among AAPs related to cardiovascular complications, long-term clinical trials are required to characterise cardiovascular complications caused by AAPs.

Other minor adverse effects related to AAPs are increased prolactin secretion, extrapyramidal side effects, sedation, dry mouth and cataract (Ucok and Gaebel, 2008).

1.10.5 Mechanisms behind AAP-induced metabolic toxicity

1.10.5.1 Dyslipidaemia

The absolute mechanism for causing dyslipidaemia in AAP-treated individuals is not fully understood, however, there is clear indication that obesity leads to the dyslipidaemia that is characterised by increased triglycerides, cholesterol, LDL and decreased HDL levels. One of the mechanisms postulated is a direct effect of AAPs on triglycerides. These drugs either stimulate the hepatic triglyceride production and secretion or may cause inhibition of the lipoprotein lipase enzyme which is necessary for hydrolysis of the triglycerides (Minet-Ringuet et al., 2007). Various studies have

suggested a potential role for transcription factor, sterol regulatory element binding proteins (SREBPs), which regulates lipids and cholesterol synthesis. It was suggested that AAPs upregulate the SREBP-1 gene in liver and adipose tissue and increased the synthesis of triglycerides (Minet-Ringuet et al., 2007, Ferno et al., 2009, Jassim et al., 2012). According to other studies, insulin resistance might play an important role in mechanism of dyslipidaemia (Yan et al., 2013, Goncalves et al., 2015). On insulin resistance, body naturally act and produce compensatory insulin from pancreas which leads to a condition called hyperinsulinemia. Physiologically, insulin regulates the processes that take place in triglyceride metabolism. In the liver, insulin blocks apolipoprotein (Apo) B release which is an important component of very low density lipoprotein (VLDL) and helps in the formation of lipids. Also, it activates SREBP1c which increase the transcription of genes necessary for fatty acid and triglyceride synthesis (Horton et al., 2002). When insulin resistance develop, there will be more release of ApoB which will result in more lipogenesis and, with the help of more ApoB, cause increased release of VLDL ultimately leading to increased plasma triglyceride levels (Lauressergues et al., 2010). Insulin has blocking action on lipolysis by acting on hormone sensitive lipase; therefore insulin resistance results in increased lipolysis leading to triglyceride breakdown and release of free fatty acids (FFA) into the circulation from adipose tissue. These FFA transport to liver and cause production of more VLDL and then the same cycle starts all over again (Adiels et al., 2005). It was also proposed that AAPs causes inhibition of enzymes involved in cholesterol synthesis leading to compensatory increase in SREBP2 which in turn trigger synthesis of genes involved in cholesterol synthesis. This was confirmed in studies where CLO and OLA increased the production of cholesterol in liver and adipose tissue (Zhang et al., 2010, Jassim et al., 2012, Polymeropoulos et al., 2009, Canfran-Duque et al., 2013).

1.10.5.2 Lipid accumulation

1.10.5.2.1 Neuroreceptors effects

The molecular mechanism of lipid accumulation by AAPs is not fully understood, however various mechanisms has been postulated which are discussed below.

It was proposed that AAPs act centrally and cause hyperphagia by acting as inhibitor at various receptors especially dopamine, histamine and serotonin (Goncalves et al., 2015). Serotonin receptor functions physiologically in a manner that it regulates food intake and increase satiety. By blocking 5HT2C receptor, it prevents satiety and leads to hyperphagia. This was confirmed in preclinical models where rodents were given 5-HT and it decreased intake of food in rodents (Balt et al., 2011). Study by Coccurello and Moles also showed that 5HT2C knock out-mice resulted in increased weight gain and feeding (Coccurello and Moles, 2010). Various studies mentioned that all AAPs have some degree of D2 receptor occupancy however the role of dopamine receptors in the context of food regulation is still controversial. Due to varying degree of drug effect on dopamine receptors, the result is confusing. However it was reported that D2 antagonism can affect eating habit (Panariello et al., 2011, Reynolds and Kirk, 2010). Preclinical studies on mice also showed involvement of H1 receptor in weight gain. Study by Masaki and colleagues showed that HI receptor knock out cause mice to eat more. However detailed studies are required to understand the absolute role of H1 receptor in weight gain (Masaki et al., 2001, Masaki et al., 2004).

Lipid accumulation and weight gain might occur due to changes in weight regulation hormones such as insulin and leptin. Leptin, apart from its peripheral action, acts on leptin receptor in hypothalamus and regulate food intake. Hyperleptinemia and leptin resistance cause increased appetite and weight gain (Melkersson and Dahl, 2004). The

role of insulin resistance and leptin will be discussed in more detail in their respective sections.

1.10.5.2.2 Peripheral effects

It was reported that weight gain can develop independently of food intake (Vestri et al., 2007). The proposed mechanism might be a direct effect of AAPs on the biology of peripheral tissues especially adipose tissue. Various *in vitro* studies have proposed an increase in lipogenesis and decrease in lipolysis as a mechanism by which AAPs caused lipid accumulation (Minet-Ringuet et al., 2007, Vestri et al., 2007). It was reported that CLO and OLA increase the differentiation of the preadipocytes which leads to triglyceride accumulation (Hemmrich et al., 2011). Ferno and colleagues reported anti-lipolysis as a mechanism causing triglyceride accumulation by AAPs, especially CLO, OLA, QTP and RISP (Ferno et al., 2009). Various *in vitro* (preadipocyte cell line (Vestri et al., 2007, Yang et al., 2007, Tsubai et al., 2017) and primary human adipocyte (Hemmrich et al., 2006, Pavan et al., 2010, Sertie et al., 2011)) studies reported that CLO and OLA have stimulating effect on adipogenesis which cause weight gain.

1.10.5.3 Insulin resistance

Adipose tissue is present in multiple areas throughout the body. Adipose tissue is not only considered as an energy storage depot but also act as endocrine organ to release various hormones and cytokines which are involved in insulin resistance. The adipocytes in adipose tissue store lipids as triglycerides in a large lipid droplet which push the nucleus to one side. One of the functions of insulin is to regulate uptake of glucose and convert into glycogen and /or triglycerides in muscle and adipose tissue. Insulin also increase the activity of lipoprotein lipase in the adipose tissue to get the lipoprotein clear from plasma. It also inhibits the function of hormone sensitive lipase in adipocytes which block the release of free fatty acids from adipocytes (Goncalves

et al., 2015, Saltiel and Pessin, 2002). Insulin after binding to its receptor, insulin receptor (IR), phosphorylates its substrate called insulin receptor substrate (IRS) which further phosphorylates several substrates. In the context of glucose regulation, phosphorylated IRS activates phosphoinositide 3-kinase (P13K) pathway activating the glucose transporter 4 and translocate it to the cell membrane where its function is to transport glucose into the cells. Insulin also has modulatory role on the activity of glucose metabolism enzymes (pyruvate kinase and hexokinase) (Correll et al., 2011).

Shulman and Niswender proposed that increase fatty acid levels may act on serine/threonine amino acid residues on IRS which ultimately make P13K unable to activate glucose transport. This leads to insulin resistance though production of insulin is normal from pancreas (Shulman, 2000, Niswender and Schwartz, 2003). Study by Morino and colleagues also reported serine phosphorylation of IRS-1 as a mechanism that leads to insulin resistance (Morino et al., 2008). Various *in vitro* studies carried out in neuronal, L6 and 3T3-L1, reported glucose uptake blockade by CLO, RISP and fluphenazine proposing that AAPs blocked glucose uptake transporter protein resulting in insulin resistance (Goncalves et al., 2015, Vestri et al., 2007). It was also reported that AAPs caused decrease in GLUT4 mRNA expression which leads to decreased movement of glucose transporter to the cell surface (Jassim et al., 2012). Another proposed mechanism is direct action of CLO and OLA on pancreatic beta cells; these drugs affect the physiological activity of pancreatic cells leading to hyperinsulinemia and insulin resistance (Reynolds and Kirk, 2010, Best et al., 2005, Melkersson et al., 2000). Various other studies showed that AAPs especially OLA caused increase in insulin secretion but decreased insulin sensitivity in response to hyperglycaemia leading to insulin resistance (Caballero, 2003, Almeras et al., 2004, Newcomer et al., 2002). Various processes like weight gain, inflammation, oxidative

stress, hyperglycaemia and hyperlipidaemia might act indirectly leading to insulin resistance (Figure 1.4).

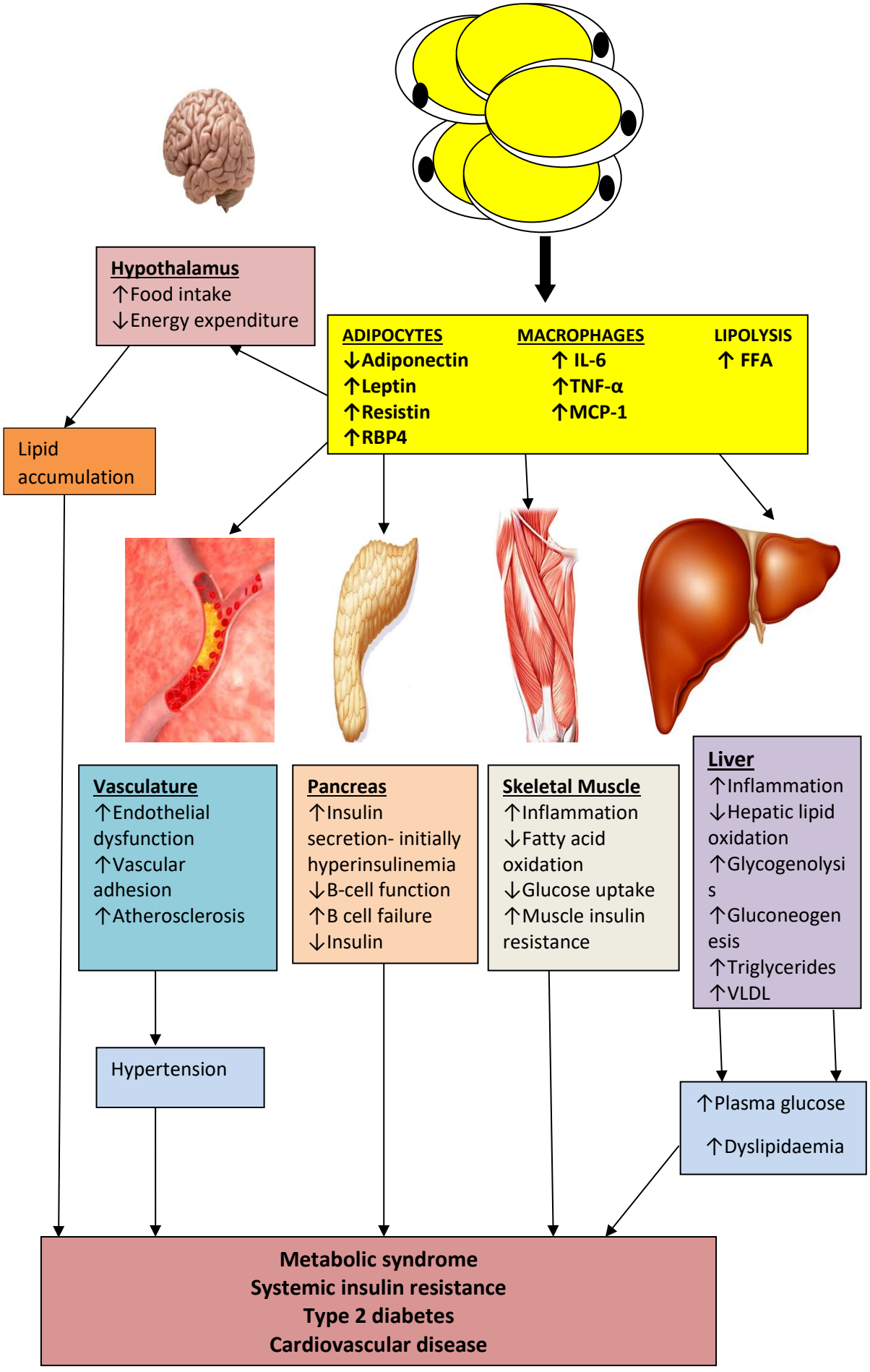


Figure 1.4 Pathophysiology of Metabolic Syndrome

1.10.5.4 Oxidative stress and mitochondrial toxicity

Oxidative stress in cells cause the production of molecules, known as reactive oxygen species (ROS) which have important role in the pathology of cells and cause cell injury or apoptosis. Preclinical studies using CLO reported decrease in antioxidant enzyme levels like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) while plasma malondialdehyde (MDA) levels were increased (Zhang et al., 2006, Agostinho et al., 2007). Study by Reinke et al in rats reported association of CLO in causing more oxidative damage compared to OLA (Reinke et al., 2004). Increased levels of antioxidant enzyme, superoxide dismutase in CLO-treated blood samples of patients have also been reported which may suggest that AAPs act by increasing ROS in cells causing metabolic toxicity (Contreras-Shannon et al., 2013).

Mitochondrial dysfunction is another mechanism suggested to cause metabolic adverse effects. Mitochondria play an important role in synthesizing adenosine triphosphate (ATP) through electron transport and oxidative phosphorylation. AAPs may act by disturbing the key metabolic enzymes of mitochondria. An *in vitro* study using neuroblastoma and lymphoblastic cells from schizophrenic patients reported alteration in mitochondrial functions by CLO-induced oxidation of mitochondrial proteins. The mitochondrial proteins are important for energy metabolism and disturbances in these proteins could result in metabolic toxicity. The oxidized proteins which were affected are enzymes such as pyruvate kinase and maleate dehydrogenase (Contreras-Shannon et al., 2013, Baig et al., 2010). Preclinical rat and mice brain studies also showed changes in mitochondrial functions (dysregulation of ATP and metabolism) and protein oxidation on using AAPs (Streck et al., 2007, Ji et al., 2009).

1.10.5.5 Inflammation

Mitochondrial dysfunction and ROS might lead to inflammatory state of the tissue. It has been documented that ROS may play a role in the production of several pro-

inflammatory cytokines (Bulua et al., 2011). In diabetic and obese patients, inflammatory cytokines like IL-6, IL-1β, monocyte chemoattractant protein (MCP) and TNF-α have been found to be elevated in their serum (Salminen et al., 2012, Lowell and Shulman, 2005). Contreras and colleagues, using 3T3-L1 preadipocyte and monocyte cell lines, reported increased levels of proinflammatory cytokines such as IL-2, MCP-1, IL-12p70, granulocyte monocyte colony stimulating factor (GM-CSF) and IL-6 when treated with CLO (Contreras-Shannon et al., 2013). This was further strengthened by another group of researchers who reported increased proinflammatory cytokines by AAPs in primary human adipocytes treated with CLO. These raised proinflammatory cytokines, especially MCP-1, could infiltrate monocytes and macrophages into adipose tissue which further increase the release of inflammatory mediators from the adipose tissue (Sarvari et al., 2014). Various clinical studies involving AAPs showed increased levels of inflammatory cytokines such as IL-10, IL-15, IL-1β, IL-17, IL-18 as well as increased leptin and decreased adiponectin secretion (Klemettila et al., 2014, Leonard et al., 2012, Goncalves et al., 2015). An important instigator of the inflammatory response to obesity is adipose tissue. It is now well recognised that adipose tissue is not only a fat storage depot but also secretes hormones, cytokines and chemokines that can function in an endocrine or a paracrine fashion. It has been reported that, more than 40% of the total adipose tissue cell content from obese rodents and humans may be composed of macrophages. These macrophages, on activation released proinflammatory cytokines which can function in a paracrine and endocrine fashion to cause decreased insulin sensitivity (Olefsky and Glass, 2010).

1.11 Role of adipokines in AAP-induced metabolic toxicity

Adipose tissue secretes biologically active adipokines, chemokines and hormone like factors. Abnormal function of these adipokines may lead to obesity, weight gain and metabolic toxicity (Balistreri et al., 2010).

1.11.1 Adiponectin

Adiponectin is an important hormone secreted by adipose tissue. Adiponectin also called as adipocyte complement-related protein of 30kDa (ACRP30 or AdipoQ). It modulates the pathway involving carbohydrate and lipid metabolism. It increases fatty acid oxidation while decreasing glucose production (Bartoli et al., 2015a, Shah et al., 2008a). Adiponectin has insulin sensitizing, anti-atherosclerotic and anti-inflammatory properties (Yamauchi et al., 2001). It act on its receptors ADIPOR1/2 and activates adenosine monophosphate (AMP)-dependent protein kinase which increase fatty acid oxidation, suppress liver gluconeogenesis and increase glucose uptake in liver (Kwon and Pessin, 2013). Arita and colleagues has reported decrease adiponectin levels in obese subjects compared to non-obese individuals (Arita et al., 1999). In a case control study, Lindsay and colleagues showed that individuals with less adiponectin levels had less insulin sensitivity and were more prone to develop T2DM (Lindsay et al., 2002). Kubota et al reported that adiponectin deficient mice showed hyperglycaemia and insulin resistance (Kubota et al., 2002). Maeda and colleagues reported insulin resistance with reduced P13 kinase activity in adiponectin deficient mice; interestingly, viral mediated adiponectin expression improved the insulin resistance and PI3K activity (Maeda et al., 2002). In a recent study, decrease in adiponectin levels were showed by CLO in an *in vitro* preadipocyte 3T3-L1 cell model (Tsubai et al., 2017). Meta-analysis by Bartolie and colleagues showed that adiponectin levels are linked with metabolic abnormalities. It was reported that

schizophrenic patients taking AAPs had lower levels of adiponectin which clearly showed correlation of adiponectin with insulin resistance and metabolic abnormality. It was further showed by Bartoli and colleagues that CLO and OLA but not RISP showed reduced levels of adiponectin (Bartoli et al., 2015b). It was suggested that adiponectin might act by directly disturbing hormonal pathways of energy homeostasis which could lead to insulin resistance and metabolic toxicity without weight gain. Decrease in adiponectin was showed in patients taking CLO and OLA; however it was reported that OLA has greater influence on adiponectin secretion compared to CLO (Lu et al., 2015). CATIE trial also showed decrease in adiponectin levels in schizophrenic patients taking AAPs (Swartz et al., 2008).

Although absolute mechanism of adiponectin increasing insulin sensitivity is not known, it was postulated that adiponectin increased the fatty acid oxidation through activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator–activated receptor (PPAR)-α. This AMPK activation, along with increased effect on fatty acid oxidation, triggers a series of reactions which increase the uptake of glucose in muscle cells. Muscle cells then convert this glucose into lactate production resulting in a reduction in liver gluconeogenesis triggering more uptake of glucose from plasma. Decreased gluconeogenesis in liver, decreased glucose levels in plasma and increased fatty acid oxidation improves the peripheral insulin sensitivity (Gil-Campos et al., 2004).

1.11.2 Leptin

Leptin is an adipocyte specific hormone released by white adipose tissue. The main function of leptin is to regulate energy balance, suppressing food intake leading to weight loss by acting on the hypothalamus (Rocha and Folco, 2011). However studies showed that leptin is not only involved in appetite control but it is capable to act as a metabolic and neuroendocrine hormone (Considine et al., 1996). It regulates glucose

metabolism, reproduction, haematopoiesis and immune system (Wauters et al., 2000). It is suggested that leptin modulate the release of insulin and thus control its secretion. This was reported in a study where leptin was given to normal fed mice which resulted in decrease in insulin levels while glucose levels increased (Kulkarni et al., 1997). Leptin levels in patients using AAPs is somewhat confusing as various studies showed mixed results; some studies mentioned higher levels of leptin (Hosojima et al., 2006, Ak et al., 2013, Potvin et al., 2015) while others showed no change in the levels (Kivircik et al., 2003, Tanaka et al., 2008). A study showed increase in leptin levels in diet-induced weight gain patients compared to lean individuals (Caro et al., 1996). The proposed mechanism could be leptin resistance as normally hyperleptinemia could cause decrease in weight gain. Study by Potvin and colleagues reported that serum leptin levels showed 318% increase in obese patients compared to non-obese while in cerebrospinal fluid (CSF) the concentration was only 30% which means enough leptin couldn't transport in obese subjects suggesting development of leptin resistance (Potvin et al., 2015).

1.11.3 IL-6

IL-6 is a cytokine released by B cells, T cells, adipocytes and monocytes and are involved in inflammatory processes and in metabolic regulation (Scheller et al., 2011). It has been postulated that IL-6 has a dual role which depends on the tissue and metabolic state. It was proposed that during exercise, skeletal muscle releases IL-6 which block the TNF-α production which is known to have role in insulin resistance and atherosclerosis. It also helps in the uptake of glucose during exercise in skeletal muscle providing glucose to the exercising muscles (Starkie et al., 2003, Makki et al., 2013). In adipose tissue and liver, IL-6 acts as a proinflammatory factor that causes insulin resistance. It was suggested that IL-6 cause insulin resistance by upregulating

suppressor of cytokine signalling 3 (SOCS3) which act on IRS1, a mediator of the insulin signalling pathway, and blocks its phosphorylation. It was also suggested that IL-6 might have role in dysregulation of fatty acid metabolism thereby leading to insulin resistance (Makki et al., 2013). IL-6 levels have been found to be increased in obese and diabetic patients having metabolic syndrome and insulin resistance (Klemettila et al., 2014, Marques-Vidal et al., 2013). Result from CATIE trial also reported elevated levels of IL-6 in patients taking antipsychotic drugs (Mori et al., 2015). In various clinical studies CLO treatment showed increase in IL-6 levels (Klemettila et al., 2014, Kluge et al., 2009, Pollmacher et al., 2000); however some studies have also showed a decrease (Sugino et al., 2009, Lu et al., 2004) or no change in IL-6 levels (Roge et al., 2012, Hemmrich et al., 2011) in schizophrenic patients.

1.11.4 Tumour Necrosis Factor-alpha

TNF-α was identified as cytotoxic substance in mice serum, which was infected with Bacillus Calmette-Guerin, as it induce haemorrhagic necrosis (Carswell et al., 1975). TNF-α was produced by monocytes, macrophages, muscle cells and adipocytes. Various roles have been documented for TNF-α in growth promotion, cytotoxicity, inflammation, metabolism and immune responses (Bradley et al., 2008). Hotamislgil and Spiegelman proposed association of TNF-α with obesity and insulin resistance (Hotamisligil and Spiegelman, 1994). Various mechanisms have been proposed for TNF-α to cause insulin resistance which includes glucose uptake blockade, downregulation of GLUT4 mRNA in adipocytes and impairment of insulin receptor and IRS-1 phosphorylation (Stephens et al., 1997, Hotamisligil et al., 1994, Hotamisligil et al., 1995). Various studies reported increase in TNF-α levels after treatment with AAPs showing its association with metabolic toxicity (Victoriano et al., 2010, Pollmacher et al., 2000, Maes et al., 1997).

1.11.5 Other Adipokines

There are other adipokines which are released by adipocytes but their role in AAP induced metabolic toxicity is still not clear. These include, resistin, ghrelin, adipsin, apelin, visfatin, vaspin, omentin, lipocalin, retinol binding protein, angiopoietin-like protein 2, chemerin, MCP-1 and plasminogen activator inhibitor (PAI)-1 (Balistreri et al., 2010, Kwon and Pessin, 2013, Makki et al., 2013).

1.12 Adipose tissue

There are three types of adipose tissue: white adipose tissue (WAT), brown adipose tissue (BAT) and, beige adipose tissue. WAT consist of adipocytes with large lipid droplet (unilocular cells) which push the other organelles to the periphery. Its major function is regulation and storage of energy in the form of triglycerides. BAT consists of smaller lipid droplets (multilocular cells) and many mitochondria which give brownish colour. Its function is thermogenesis and generation of heat (Seale et al., 2009). Beige adipocytes are part of WAT which on stimulation acquire phenotype similar to brown adipocytes. WAT are present mostly in adults, where majority is present under the skin called as subcutaneous adipose tissue while the other is deposited around viscera such as mesenteric, perirenal, omental, mediastinal and pericardial while BAT is present mostly in infants but evidence showed existence of BAT in adults as well. They are present mainly in supraclavicular, suprarenal, interscapular and around aorta (Nedergaard et al., 2007). Adipose tissue consist of preadipocytes, adipocytes, fibroblasts, inflammatory cells and mesenchymal stem cells (Gustafson et al., 2007). Adipogenesis involves a highly complex but organised system of gene expression. Understanding of complexity of adipogenesis is very

important which also help in development of new strategies for improving adipose tissue related diseases such as metabolic toxicity (Lowe et al., 2011).

1.12.1 Adipocyte stem cells

Green and Kehinde established immortal fibroblast cell lines that differentiated in to mature adipocytes on hormonal inducers. These known as 3T3-L1 and 3T3-F442A; isolated from non-clonal swiss mouse 3T3 cells which are widely used for *in vitro* experiment. Later on, detailed research resulted in development of other preadipocyte cell lines like 1246, Ob1771, TA1 and 30A5 though there were little differences in their differentiation condition but they act similar as 3T3-L1 and 3T3-F442A (Rosen et al., 2000, Ntambi and Young-Cheul, 2000). Adipocytes can now be developed from pluripotent mesenchymal stem cells (MSCs) which can be isolated from adipose tissue and muscle. These MSC can be differentiated into cell types like adipocytes, chondrocytes, myocytes and osteoblast. Further research also separated stem cells from adipose stromal vascular fraction but their capacity to develop into adipocytes are found to be variable both *in vitro* as well as *in vivo* (Lowe et al., 2011, Rodeheffer et al., 2008). Also cell surface markers has been proposed to contain stromal cells that differentiate into adipocytes *in vivo* which include CD34+, CD24+, a7, Scal+, PDGFR alpha+ but these cells show limited adipogenic activity which need to be further explored (Tang et al., 2008, Rodeheffer et al., 2008). Various cellular models, which are sources of adipocytes have been presented in diagram below (Rosen and MacDougald, 2006).

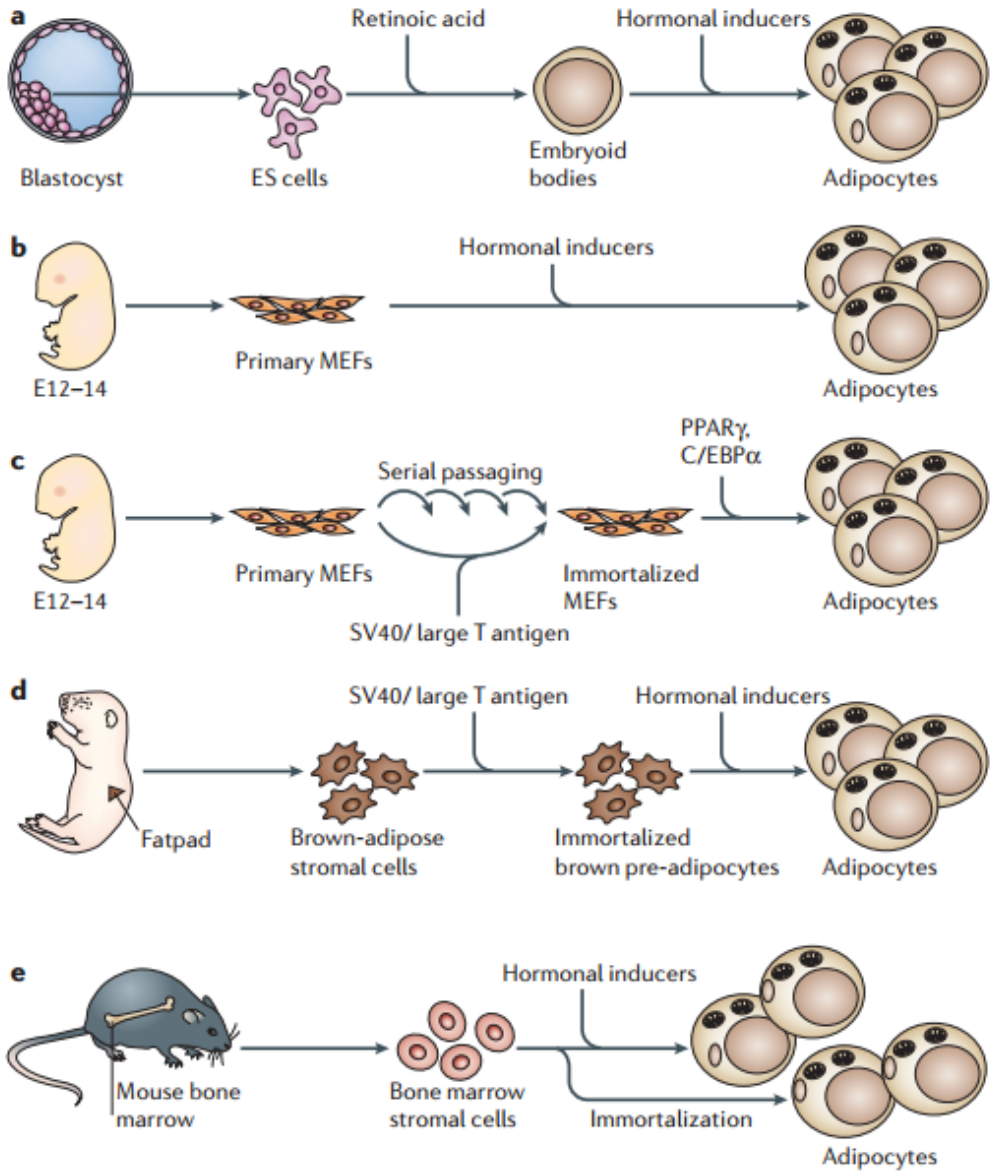


Figure 1.5 Various models used to study adipogenesis (adopted from (Rosen and MacDougald, 2006). a) Embryonic stem (ES) cells differentiated directly into adipocytes, under influence of retinoic acid and pro-adipogenic hormones. (b) Mouse embryonic fibroblasts (MEFs), which can be isolated after disaggregation of embryos at embryonic day (E)12–14, can be differentiated to adipocytes (c) or can be immortalized by serial passaging or introduction of the SV40 large T antigen prior to differentiation. Most immortalized MEF lines do not differentiate unless a pro-adipogenic transcription factor such as proliferator-activated receptor γ (PPARγ) or CCAAT-enhancer-binding protein α (C/EBPα) is introduced. (d) Brown-adipose stromal cells from newborn mice can be immortalized using SV40 large T antigen, and differentiate efficiently on hormonal treatment. (e) Multipotent precursor cells isolated from several adult tissues including adipose tissue, skeletal muscle and bone marrow provide another source of that has been isolated from bone marrow cells, which are particularly useful for studies on mesenchymal-cell-fate decisions.

1.12.2 Adipogenesis

Better understanding of adipogenesis could contribute towards the development of potential therapeutic strategies for metabolic diseases such as obesity and metabolic syndrome. Improvement in adipocyte functions and replacement of poorly functioning adipocytes could be beneficial in metabolic disease (Lowe et al., 2011). Adipogenesis comprises of various stages involving transcription factors, cell cycle proteins and gene expression regulation which leads to development of mature adipocyte (Lefterova and Lazar, 2009). Adipogenesis comprises of two main phases:

Determination phase: In this phase multipotent MSC converted into preadipocytes which then go through growth phase followed by growth arrest and clonal expansion. In growth arrest, preadipocytes withdraw from cell cycle before undergoing adipose conversion. This is necessary for adipocyte differentiation. In clonal expansion, preadipocytes goes through one or more cycle of mitosis before entering into terminal differentiation phase.

Terminal differentiation phase: In this phase, the preadipocyte takes on the characteristic of mature adipocyte ready to perform its physiological actions. It acquires the machinery which is necessary for insulin action, lipid transport and synthesis and the secretion of adipocyte-specific proteins (Umek et al., 1991, Lane et al., 1999, Ntambi and Young-Cheul, 2000, Spiegelman et al., 1983). These phases are controlled by complex network of factors and genes which are briefly described below. Various signals played role in the initiation of adipogenesis from conversion of stem cells to adipocytes. These include fibroblast growth factor, interleukin 17, insulin, insulin like growth factor 1 (IGF-1), transforming growth factor, thyroid hormones, glucocorticoids, mineralocorticoids, PPARγ and bone morphogenetic proteins (Lowe et al., 2011). Insulin has promising effect on adipogenesis. Downstream component of the insulin/IGF-1 signalling cascade are important for adipogenesis. Downstream effectors of insulin action cascade, such as PI3K and protein kinase B (PKB) have

been reported to be involved in adipogenesis (Kim and Chen, 2004). Glucocorticoids (GC) are also potent inducers of adipogenesis. GC receptors are present in human preadipocytes and it activates the expression of C/EBPδ and PPARγ (Wu et al., 1996). Some fibroblast growth factors such as FGF1, 2 and 10 showed adipogenic activity on human preadipocytes and, their neutralization inhibits adipogenesis (Hutley et al., 2004).

A variety of cytokines including TNF-1, IL-1 and many proinflammatory molecules have been found to inhibit adipogenesis in most cultured preadipocyte lines (Ohsumi et al., 1994). In addition several growth factors are potent inhibitors of adipogenesis, including platelet- derived growth factor and endothelial-derived growth factor (Hauner et al., 1995). The inhibitory effect is supposed to be mediated by activation of mitogen-activated protein kinases (MAPK). These kinases phosphorylate PPARγ2 at Ser112 in the amino-terminal domain and inhibit its adipogenic activity (Rosen et al., 2000).

Signalling pathways also play an important role in adipogenesis. One of them is WNT signalling, the suppression of which is necessary for adipogenesis (Prestwich and Macdougald, 2007). Another signalling pathway, called as hedgehog (HH) which inhibits adipogenesis, has been proposed to have a role in adipogenesis (Pospisilik et al., 2010). The absolute mechanism involved is not clear however role of HH and WNT signalling receptors has been proposed. It was suggested that HH and WNT signalling receptors, present on the primary cilia in differentiating adipocytes, become impaired during adipogenesis steps which leads to over expression of PPARγ gene. This over expression resulted in increased adipogenesis which might end up to obese phenotype (Marion et al., 2009). Other pathways which have been reported to be involved in adipogenesis are SMAD signalling (Tan et al., 2011, Marchildon et al., 2010), ribosomal protein S6 kinase 1(S6K1) (Carnevalli et al., 2010), the Janus kinase-

signal transducer and activator of transcription 3 pathway (JAK-STAT3) (Zhang et al., 2011), glutathione, AKT and inositol pyrophosphate pathway (Chakraborty et al., 2010). It was also reported that transcriptional regulators like retinoblastoma protein (RB), p53, zinc-finger protein 423 and proto-oncoprotein MAF are controlled by the above mentioned pathways (Molchadsky et al., 2008, Nishikawa et al., 2010, Calo et al., 2010, Gupta et al., 2010). Any imbalance disturbs the pathways which ultimately halt process of adipogenesis. Some studies suggested cell shape as a factor influencing adipogenesis. It has been shown those rounded mesenchymal stem cells are more likely to become adipocytes (Kilian et al., 2010, Feng et al., 2010). Role of extracellular remodelling has also been postulated in adipogenesis which showed that impairment of membrane-bound matrix metalloproteinase (MMP14) leads to defective adipogenesis (Chun et al., 2006).

1.12.2.1 Role of Transcriptional factors during adipogenesis

Adipocyte differentiation involves a regulated set of gene expression events and understanding the transcriptional network is of fundamental importance. Once the adipogenesis process started, it over expressed adipogenic early key regulators CAAT/enhancer-binding proteins (C/EBP) β and δ. These factors target the genes encoding PPARγ, considered as master regulator of adipogenesis, CEBP α and SREBP1 which regulates lipogenic genes (Rosen and MacDougald, 2006, Payne et al., 2009). PPARγ then activates genes encoding C/EBPα through positive feedback mechanism. PPARγ and C/EBPα then over express genes which have a role in lipogenesis, lipolysis and insulin sensitivity including fatty acid binding protein (FABP4), lipoprotein lipase (LPL), GLUT 4, perilipin, lipin, leptin and adiponectin.

PPARγ, a member of the nuclear hormone receptor superfamily, heterodimerize with retinoid X receptor to bind DNA and be transcriptionally active. PPARγ has 2 isoforms generated by alternative splicing. PPARγ2 is more abundant in fat cells compared to PPARγ1 which is abundant in colonic epithelium and macrophages (Mansen et al.,

1996).PPARγ is not only important for adipogenesis but is also required for maintenance of differentiated adipocytes. Adenoviral gene transfer of a dominant-negative PPARγ into mature 3T3-L1 adipocytes resulted in decrease in cell size and expression of adipocyte markers (Tamori et al., 2002). *In vivo* knock down of PPARγ in differentiated adipocytes resulted in adipocyte death followed by generation of new adipocytes (Imai et al., 2004).

Several C/EBPs including C/EBPα, β, γ, and δ are expressed in adipocytes. Regulated expression has been observed for C/EBP family during adipogenesis and studies indicated that these proteins have profound impact on development of adipocytes (Rosen et al., 2000, Lowe et al., 2011). It has been reported that C/EBP β and δ are involved in early differentiation of adipocytes while C/EBPα is induced later, slightly preceding the induction of adipocytes end-product genes. It was also proposed that C/EBP β and δ promote adipogenesis by inducing C/EBPα and PPARγ (Darlington et al., 1998).

SREBP1c is a pro-adipogenic basic helix-loop-helix (bHLH) transcription factor that induce PPARγ expression. SREBP1c role in adipogenesis was proposed by the observation that the mRNA expression of SREBP1c was induced dramatically as 3T3-L1 preadipocyte cell line was stimulated to undergo differentiation. SREBP1c overexpression in the presence of hormonal inducers of differentiation resulted in increased adipocyte marker expression and lipid accumulation in 3T3-L1 cells compared to control cells (Kim et al., 1998b). It was proposed that SREBP1c produced some factors that enhances PPARγ activity (Kim et al., 1998b). Others proposed that SREBP1c itself activated PPARγ and then mutually acted together to increase adipogenesis (Fajas et al., 1999, Kim et al., 1998a). SREBP1c also regulate various genes linked to fatty acid and triglyceride metabolism that include acetyl-CoA carboxylase, fatty acid synthase and glycerophosphate acyltransferase (Rosen et al.,

2000). Other transcription factors that are linked to adipogenesis are orphan nuclear receptor, nuclear factor of activated T cells (NFAT), high-mobility group proteins and cyclic AMP response element binding protein (Rosen et al., 2000).

There have been factors reported to act as adipogenic as well as anti-adipogenic at different stage of adipogenesis to regulate this highly complex network. One of the example is nuclear receptor, chicken ovalbumin upstream promoter-transcription factor 11(COUP-TF11) which promote preadipocyte lineage by blocking WNT signalling, however it was reported to be act as inhibitor of C/EBPα and PPARγ thus inhibit adipogenesis (Xu et al., 2008, Li et al., 2009). It has also been reported that the factors which regulate adipogenesis also affect its activity during adipogenesis. To explain this, it has been reported that Sirtuin (SIRT2) act on forkhead box O1 (FOXO1) and blocked its acetylation and phosphorylation which ultimately increase its nuclear localization. This increase in FOXO1 blocked genes encoding PPARγ thus inhibits PPARγ leading to impaired adipogenesis. Another example of PPARγ inhibitor is retinol binding protein1 (RBP1) (Zizola et al., 2010, Jing et al., 2007, Picard et al., 2004). Phosphorylation has also been reported to be another factor which regulates adipogenesis. One of such example is CDK5 which phosphorylate Ser273 of PPARγ gene leading to decrease expression of its target genes in adipocytes (Choi et al., 2010). Others excitatory regulators includes early growth response-2 (EGR2) (White and Stephens, 2010), early B cell factor 1 (EBF1) (Jimenez et al., 2007), kruppel like factor 4) (KLF4) (Birsoy et al., 2008) while inhibitory factors include KLF3, C terminal binding protein 1 and 2 (CTBP) (Sue et al., 2008), interferon regulatory factor (IRF) 3 and 4 (Eguchi et al., 2008) and globin transcription factor 2 and 3 (GATA 2 and 3) (Tong et al., 2005).

In short, adipogenesis involved various pathways which regulate numerous genes at cellular level.

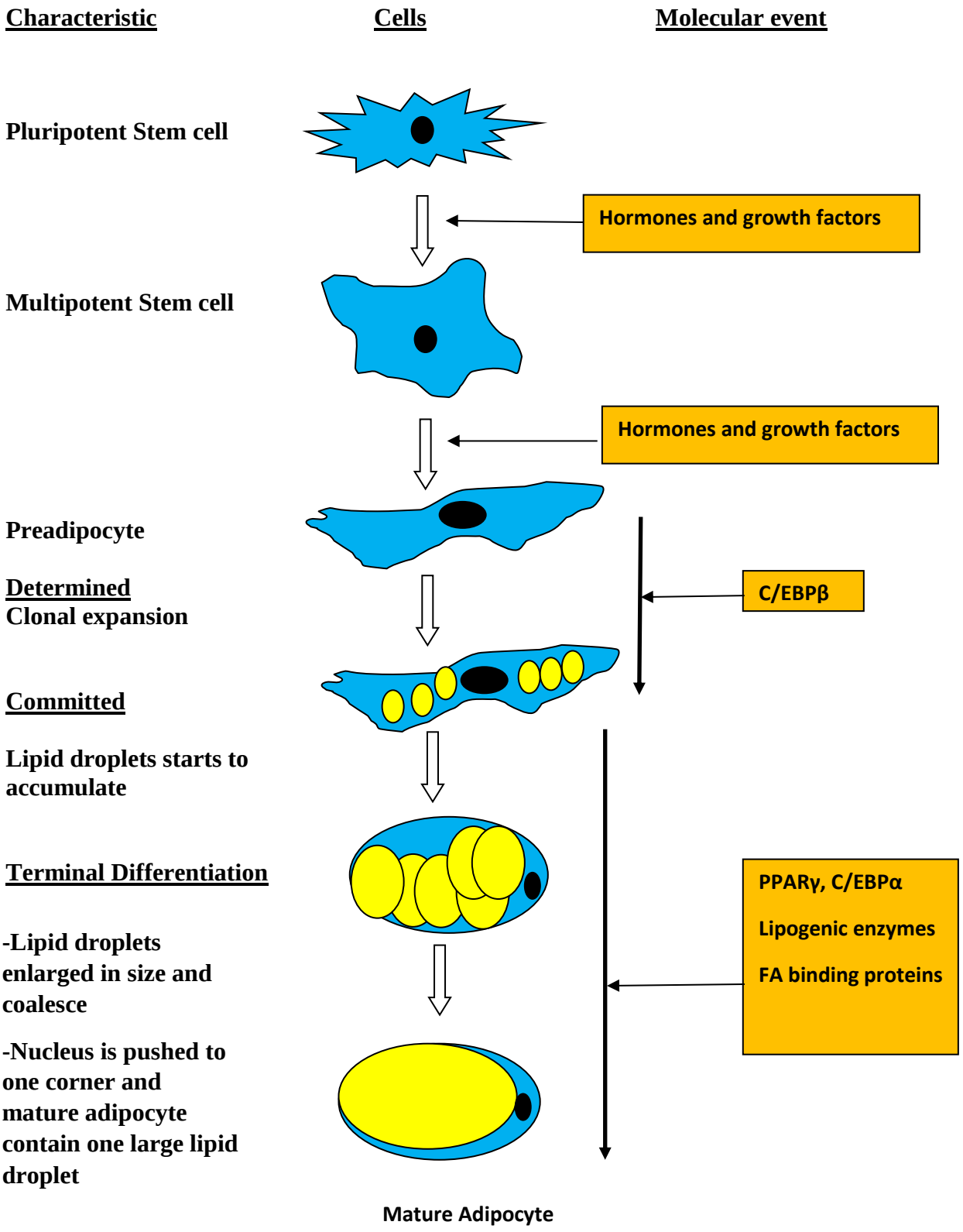


Figure 1.6 Stages of Adipogenesis

1.13 AAPs induced gene dysregulation in adipocytes

Various transcriptional factors play role in differentiation of preadipocytes. Three most important transcriptional factors are PPARγ, C/EBPα and SREBP (Yang et al., 2009, Rosen et al., 2000). SREBP plays an important role in fatty acid metabolism and also regulates genes involved in lipid synthesis. Upregulation of SREBP has great effect on downstream genes and differentiation markers and increases their expression such as PPARγ, C/EBP α, FABP4 and fatty acid synthase (FAS). Various studies reported increased expression of SREBP, PPARγ and C/EBPα along with lipid accumulation by AAPs showing role of these transcription factors in metabolic toxicity (Hu et al., 2010, Yang et al., 2007). This was also showed in a study by Li and colleagues reported increase expression of SREBP and downstream genes by OLA (Li et al., 2016). Study by Yang and colleagues showed that CLO had no effect on PPARγ and C/EBPα however they proposed SREBP as the main transcription factor which played role in lipid accumulation and weight gain (Yang et al., 2009). *In vitro* study by Raeder and colleagues reported increase expression of SREBP by CLO while ZIP did not show any change in SREBP expression which showed the association of SREBP gene in metabolic toxicity caused by AAPs (Raeder et al., 2006). However *in vitro* primary human adipocytes study by Sarvari and colleagues showed changes in various genes including INSR, PPARα, LEP, ADIPOQ and SIRT1 after giving AAPs suggesting involvement of many genes in metabolic toxicity. Their study also reported that CLO increased PPARγ expression while ZIP showed no change. AAPs also did not show any change in SREBP1 expression in a study by Sarvari and colleagues (Sarvari et al., 2014). Change in Inflammatory genes might have role in metabolic toxicity. It has been shown that AAPs caused increase expression of nuclear factor kappa (NF-KB1) and proinflammatory cytokines such as TNF-α, IL-1β, IL-8 and MCP-1. This increase may cause macrophages to infiltrate to adipose tissue and cause

inflammation which leads to metabolic toxicity (Sarvari et al., 2014). This involvement was also showed in another study where OLA caused increased expression of TNF-α (Victoriano et al., 2010). It was proposed that increasing TNF-α expression or secretion might have effect on GLUT4 transporter and IRS1 leading to reduce insulin activity resulted in insulin resistance (Sarvari et al., 2014).

Lipin (LPIN) gene might have important role in metabolic toxicity. The LPIN gene encodes three proteins, 1, 2, and 3. It was first isolated from fatty liver dystrophy mutant mouse strain. LPIN1 is abundant in adipose tissue, skeletal muscle and testis while it is also found in kidney, brain, heart and liver. During triglyceride synthesis, LPIN1 act by converting phosphatadate into 1, 2-diaacylglycerol (Reue, 2009). The LPIN1 is essential for development of adipocytes.

LPIN1 gene is a late adipogenic differentiation marker without which adipocytes cannot fully differentiate and mature as well. It was proved by knock down of LPIN1 gene in 3T3-L1 preadipocytes which inhibited adipocyte differentiation whereas increased expression induced adipocyte differentiation. It has been reported that LPIN1 worked as co-activator of PPARγ2 and regulates the network between PPARγ2 and C/EBPα activating the genes required for adipogenesis. Various preclinical studies on animals showed that lack of LPIN1 gene in mice, resulted in failure to develop mature adipocytes leading to lipodystrophy and fatty liver while transgenic mice with increased LPIN1 expression resulted in hypertrophic adipocytes (Koh et al., 2008, Reue and Zhang, 2008, van Harmelen et al., 2007). However in human, the relationship of LPIN1 expression with respect to obesity and insulin sensitivity is complex. Various studies have shown the downregulation of LPIN1 gene in obese and diabetes mellitus 2 subjects (van Harmelen et al., 2007, Yao-Borengasser et al., 2006) while other studies showed no correlation between LPIN1 and insulin sensitivity (Miranda et al., 2010). The mechanism of decreased LPIN1 expression in obese

patients is not known however various factors have been proposed which might affect the expression of lipin1 which include environmental factor, genetic factors, effect of drugs, abnormal adipocyte function and posttranslational modification (Yao-Borengasser et al., 2006).

1.14 Genetic predisposition related to AAP-induced metabolic toxicity

Genetic factors are considered to be strongest risk factors for AAP-induced metabolic toxicity. Several studies have suggested association between genetic variants and AAP-induced metabolic toxicity (Devlin and Panagiotopoulos, 2015, Muller et al., 2012, Ujike et al., 2008). Pharmacokinetically, AAPs are metabolized by cytochrome (CYP) 450 isoenzymes. This means poor activity of CYP450 leads to more levels of AAPs causing more adverse effects. Study by Lett and colleagues showed that polymorphisms in CYP P450 2D6 gene (CYP2D6) might be involved in AAP-induced weight gain. This gene is involved in RISP metabolism and it was reported that the T-allele carrier of the 188C/T variant have decreased CYP2D6 function (Lett et al., 2012). Study by Lane and colleagues on Chinese schizophrenic patients undergoing RISP treatment also showed association of 188T allele of CYP2D6 with increased weight gain (Lane et al., 2006). The efficacy of pharmacokinetics in AAPs-induced weight gain still undetermined and more research is needed in this part. Pharmacodynamically, serotonin receptor gene HTR2C was studied most to understand the mechanism behind AAPs-induced metabolic toxicity. Various studies showed association of rs3813929 (C/T) promoter SNP (-759C/T) of the HTR2C gene with increase weight gain (Ryu et al., 2007, Laika et al., 2010, Lane et al., 2006). Another SNP in HT2RC gene (rs1414334) was also reported to be associated with metabolic syndrome in patients taking CLO and RISP (Mulder et al., 2009). Various studies also reported association of polymorphism in HTR2A gene with weight gain in patients treated with OLA and RISP (Ujike et al., 2008, Lane et al., 2006).

Polymorphism in dopamine receptor gene also showed association with weight gain. It was reported that SNPs (rs4436578, rs1799732 and rs1079598) of DRD2 gene showed association in schizophrenic patients using CLO, OLA and RISP (Lane et al., 2006). Numerous studies showed association of various genes, their SNPs and alleles with AAPs-induced weight gain which include; MTHFR and its SNP (rs1801133) (Srisawat et al., 2014, Shams and Muller, 2014); leptin gene and its SNP (rs7799039) (Nurmi et al., 2013, Brandl et al., 2012); glucagon like peptide GLP-1 gene (rs3799707 and rs13429709) (Brandl et al., 2014a) and Melanocortin-4 receptor (MC4R) and its SNP (rs17782313 and rs11872992). Study by Gonclaves and colleagues showed association of mitochondrial genes with AAPs-induced weight gain and metabolic toxicity. Their study reported significant association between rs6435326, rs1053517 and rs1801318 in NADH: Ubiquinone Oxidoreductase Core Subunit S1 (NDUFS1) gene and weight gain (Goncalves et al., 2014).

However some studies did not show any gene association with AAPs induced weight gain. Godlewsky and colleagues showed no association of histamine H receptor (HIHR) gene with AAPs-induced weight gain. Their group studied five SNPs, rs2067466, rs2067467, rs2067469, rs2067468 and rs2067470 but no association was found between these SNPs and OLA induced weight gain (Godlewska et al., 2013). Study by Lett et al also reported no association of rs3813929 SNP in HTR2C gene (Lett et al., 2012). Controversial results are also reported with various genes as some study showed association with weight gain while other did not show association with the same gene. These include adiponectin (ADIPOQ) and PPARγ. Herken and colleagues showed association of PPARγ with weight gain while Brandl and colleagues reported no association of PPARγ and ADIPOQ gene with weight gain (Kohlrausch, 2013, Herken et al., 2009, Brandl et al., 2014b).

Genome wide association studies (GWAS) showed association of many genes including HTR2C, leptin, fat mass and obesity-associated (FTO), DRD2, TNFα, Synaptosomal-associated protein 25 (SNAP-25), MC4R, and INSIG2 in metabolic toxicity caused by antipsychotic drugs. The top finding emerge to be association of SNP (rs1568679) of Meis homeobox2 (MEIS2) gene for increase in waist and hip circumference in patients given RISP. This gene is important for the development and function of pancreas which might have link between adiposity and diabetes. This GWAS also reported association of SNP (rs13224682) of protein kinase cyclic adenosine monophosphate-dependent regulatory type 11 beta (PRKAR2B) gene with triglyceride increase in CLO-treated patients. This gene controls the cellular effects of cAMP so it regulates many signalling pathways (Adkins et al., 2011, Correll et al., 2011). Further studies are required to validate these genes and their association with metabolic toxicity so that physician can apply personalised medicines (AAPs) to schizophrenic patients (Gressier et al., 2016, Lett et al., 2012).

1.15 Aim of the thesis

AAPs are the mainstay of treatment in schizophrenia however their use is impeded by the high frequency of metabolic adverse effects like insulin resistance, weight gain with abdominal obesity, diabetes mellitus, dyslipidaemia and essential hypertension observed in these patients. AAPs lead to cardiovascular complications which badly affect the quality of life resulting in greater mortality rate compared to general population (Scigliano and Ronchetti, 2013). Various mechanisms have been proposed for the metabolic toxicity caused by AAPs; these include direct effect on adipose tissue, insulin resistance, inflammation, oxidative stress, hormonal effect and CNS receptor effects. But none of the mechanisms have shown the role of any specific system involved in metabolic toxicity. The main purpose of this work is to improve

our understanding of AAP-induced metabolic toxicity using in vitro adipocyte models and to test therapeutic strategies than can arrest or reverse AAP-induced metabolic toxicity. Specific aims of this study are to understand the role of AAPs in causing metabolic toxicity by:

1) Characterisation of the effect of specific AAPs on adipogenesis and adipokines using *in vitro* murine adipocyte models and primary human adipocytes.

2) Understanding the molecular mechanisms that are responsible for AAPs-induced metabolic toxicity.

3) Investigation of the adjuvant drugs potential to reverse the adipotoxicity caused by AAPs.

4) Characterisation of the lipidomic signature of AAPs in primary human adipocytes.

Chapter 2

Effect of Atypical antipsychotics on adipogenesis using *in vitro* murine adipocyte model (3T3-F442A)

2.1 Introduction

AAPs are second-generation antipsychotics commonly prescribed for the treatment of psychiatric disorders including schizophrenia and psychosis (Sarvari et al., 2014). As compared to first-generation antipsychotics, AAPs show fewer extrapyramidal adverse effects. However, the long-term use of these drugs is associated with potentially serious metabolic adverse effects. The major side effects of AAPs are weight gain and associated metabolic disorders such as insulin resistance, obesity, impaired glucose tolerance, T2DM, dyslipidemia and essential hypertension. These metabolic abnormalities lead to the development of cardiometabolic disease (Nasrallah, 2008). The risk of metabolic toxicity differs between different AAPs. It was concluded that CLO and OLA constitute the highest metabolic risk; QTP, RISP, and AMI intermediate; while ARI and ZIP were considered to pose a lower risk (Wang et al., 2013). The exact mechanism behind AAP-induced metabolic adverse effects is not yet known however it is believed that these drugs cause these effects by acting centrally and peripherally. Various studies showed that AAPs disturbed histamine, serotonin and noradrenergic signalling targeting central nervous system pathways involved in food intake. This suppresses satiety leading to increasing eating habits which lead to weight gain and its complications (Balf et al., 2008, Coccurello and Moles, 2010, Masaki et al., 2004). One of the suggested mechanism is the direct effect of AAPs on adipocytes. White adipose tissue is an important endocrine organ with important functions. The major constituent of adipose tissue is the adipocyte. The main function of fat cells is to maintain glucose and lipid homeostasis and store and release energy when required. Surplus energy is stored as triglycerides in the form of lipid droplets (Lefterova and Lazar, 2009). Some studies mentioned the involvement of subcutaneous adipose tissue as a key organ to affect metabolic hormones. It was proposed that AAPs act directly on the adipose tissue increasing adipogenesis which

ultimately leads to increased lipid accumulation and weight gain (Minet-Ringuet et al., 2007, Hemmrich et al., 2011). However another study showed no direct effect of AAPs on fat cell formation and highlighted the central role of AAPs in causing metabolic toxicity (Hauner et al., 2003). Other proposed mechanisms include adipose mitochondrial dysfunction which disturbs electron transport chain pathway (Ji et al., 2009, Streck et al., 2007), increased production of reactive oxygen species (Miljevic et al., 2010) or genetic factors (Lett et al., 2012). Other researchers reported the role of inflammation in AAP-induced metabolic adverse effects. According to these studies, AAPs caused metabolic toxicity by increasing the release of proinflammatory cytokines like IL-6 and TNF-α which caused phosphorylation of insulin receptors leading to insulin resistance; one of the major features of metabolic toxicity (Makki et al., 2013, Klemettila et al., 2014, Pollmacher et al., 2000). Various clinical trials found CLO and OLA to increase TNF-α and IL-6 levels after AAP treatment which further implicates their role in metabolic adverse reactions (Klemettila et al., 2014, Kluge et al., 2009). Adipose tissue also secretes adiponectin, an adipose-derived hormone which modulates the pathway involving carbohydrate and lipid metabolism. In obesity, adipokine secretion and expression is disturbed along with dysregulation of glucose and lipid metabolism leading to metabolic abnormalities. Meta-analysis of plasma adiponectin levels in schizophrenic patients showed decrease in adiponectin levels by CLO and OLA. Decreased adiponectin levels may lead to obesity, insulin resistance and T2DM (Bartoli et al., 2015c).The exact mechanism causing decreased adiponectin levels are not fully explored however the role of adiponectin has been proposed as a link in metabolic abnormality (Bartoli et al., 2015b, Wang et al., 2013). During adipogenesis, preadipocytes, a fibroblast-like cell, differentiates into mature adipocytes. This process takes various stages which involve various transcriptional factors which includes PPARγ, C/EBPs, SREBP and LPIN1 (Yang et al., 2009).

PPARγ, a master regulator of adipogenesis develops and maintain fat cells and their capacity to store lipids (Medina-Gomez et al., 2007). The importance of PPARγ was demonstrated by targeted gene knockout strategy in mice; this resulted in the failure of development of adipose tissue (Evans et al., 2004). It has been shown that AAPs caused a stimulatory effect on PPARγ; this might have a link with AAP-induced weight gain and AAP-mediated increase in adipocyte lipid accumulation (Sarvari et al., 2014). However, another study showed no role of PPARγ in weight gain (Brandl et al., 2014a). Another important transcription factor required for normal adipocyte differentiation is lipin 1. LPIN1 gene knockdown resulted in inhibition of adipocyte differentiation and it increases the function of PPARγ2 through direct interaction with and activation of PPARγ2 (Kim et al., 2013, Koh et al., 2008, Yao-Borengasser et al., 2006). The relationship of LPIN1 expression with obesity or metabolic syndrome is complex. Some studies have shown downregulation of *LPIN1* gene in obese and T2DM subjects (van Harmelen et al., 2007, Yao-Borengasser et al., 2006) while others have showed no relation between LPIN1 and metabolic syndrome (Miranda et al., 2010). Under normal conditions, adipocytes store excess triglycerides while in obese subjects, adipocytes may reach maximum capacity of their fat storage. Due to this, lipid may be stored in ectopic sites such as liver, skeletal muscle, and heart which disturb their physiological functions, ultimately leading to insulin resistance. Increased lipolysis from excess triglycerides in adipose tissue resulted in higher levels of FFA (Schenk et al., 2008). This FFA then caused glucose intolerance resulting in insulin resistance. This was further supported by Boden and Kelly's study showing development of insulin resistance induced by FFA (Boden, 1997, Kelley et al., 1993).

2.1.1 Rationale for the study

Various mechanisms, some of which are contradictory, have been postulated for the pathogenesis of AAP-induced metabolic toxicity so far. CLO and OLA has been suggested to cause metabolic adverse effects compared to other AAPs (Swartz et al., 2008, Kluge et al., 2009, Young et al., 2015); this could be as a result of their effects on adipogenesis (Hemmrich et al., 2011, Hu et al., 2010); or their effect on inflammatory cytokines (Pollmacher et al., 2000, Makki et al., 2013); or other adipokines such as adiponectin (Wang et al., 2013, Bartoli et al., 2015b); or a mixture of these effects. Our study used a 3T3-F442A murine preadipocyte cell line model to investigate the effect of AAPs on lipid accumulation, adipogenic transcription markers, and adipocytokine release. This study primarily focussed on three AAPs: CLO and OLA, which are considered to result in metabolic adverse effects; and ARI, an AAP with little metabolic safety liability.

2.1.2 Hypotheses

The hypotheses tested in this chapter were;

1) AAPs cause lipid accumulation by a direct effect on adipogenesis and thereby lead to weight gain and ultimately metabolic toxicity.

2) AAPs upregulate the expression of adipogenic transcription factor PPARγ and other important transcription factors such as LPIN1.

3) AAPs cause changes in the release of adipocytokines which could be mechanistically important in the development of insulin resistance and metabolic adverse effects.

2.1.3 Aims and objectives

The aims and objectives were;

1) To investigate AAP-induced metabolic toxicity using an *in vitro* murine adipocyte model by the assessment of:

a. adipocyte function and gene transcription;
b. adipocyte-secreted markers and
c. inflammatory cytokines

2.2 Methods

2.2.1 Materials

AAPs, CLO and OLA were purchased from Sigma-Aldrich, St. Louis, MO, USA while ARI and lopinavir (LPV) were purchased from Santa Cruz Biotechnology, Texas (TX), USA. Methanol, ethanol, dimethyl sulfoxide (DMSO), trypsin, dimethylformamide, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT), insulin from bovine pancreas, radioimmunoprecipitation (RIPA) lysis buffer, sodium dodecyl sulphate (SDS), Ethylenediaminetetraacetic acid (EDTA), chloroform and Oil Red O stain were purchased from Sigma-Aldrich, St. Louis, MO, USA. Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), Hank's balanced salt solution (HBSS), rat tail collagen I, reverse transcription kit, Tri Reagent solution for RNA, FAM labelled PPARγ and lipin1, VIC labelled β-actin TaqMan gene expression assays, TaqMan gene expression master mix, isopropanol, enzyme-linked immunosorbent assay (ELISA) kits for adiponectin, IL-6 and TNF-α were purchased from Thermo Fisher Scientific, TX, USA. PPARγ (catalogue number: 2443) antibody was purchased from Cell signalling technology, Massachusetts (MA), USA, lipin1 (catalogue number: sc-98450) and β-actin (catalogue number sc-47778) were purchased from Santa Cruz Biotechnology, TX, USA. FFA assay kit, detergent compatible (DC) protein assay kit and Simple Western assays were purchased from Abcam, Cambridge, UK, Bio-Rad, California (CA), USA and ProteinSimple, CA, USA respectively. 12 well and 6 well polystyrene plates, T175, T75 flasks and other cell culture materials were purchased from Thermo Fisher Scientific, TX, USA. 3T3-F442A murine preadipocyte cell line was available in-house in the Wolfson centre for Personalised Medicine, Department of Molecular and Clinical Pharmacology, University of Liverpool, UK.

2.2.2 Cell Culture

Preadipocytes were cultured and maintained in T75 flasks in DMEM containing 10% FBS. Once confluent (75-80%), cell culture media was discarded and cells were washed carefully with HBSS. After washing, 5ml of trypsin was added to the flasks and incubated for 5 minutes to dislodge the cells from the surface of the flasks. After incubation at 37 ^{O}C for 5 minutes, the cells were detached by gentle tapping of the bottom of the flask. The cell suspension was poured into a universal tube containing 10ml of DMEM to neutralise the trypsin. The tubes were centrifuged at 1000rpm for 5 minutes at room temperature to obtain the cell pellet. The supernatant was discarded once centrifugation finished and the cell pellet was suspended in 3-5ml of DMEM depending upon the size of the pellet. Cell counting was done by mixing 10µl of the cell suspension with 10µl of trypan blue dye. Then 10µl mixture of cells and dye was loaded into a Countess Cell chamber slide (Thermo Fisher Scientific, TX, USA) which was then counted by using a Countess Cell counter (Thermo Fisher Scientific, TX, USA). One million cells were suspended in adequate amount of media and sub-cultured in new T75 flasks for maintenance of cell line. The remaining cells were either frozen or cultured in collagen- coated plates as required.

2.2.2.1 Freezing of cells

Once the required number of cells were separated for maintenance in a T75 flask or collagen-coated plates, the universal tube containing media with cells was centrifuged at 1000rpm for 5 minutes to obtain the pellet. The supernatant was discarded and freezing medium was added according to the cell number (1 ml freezing medium per 1 million cells). After mixing, this mixture was added into cryovials. These cryovials were then kept in Mr Frosty, a container which freeze at the rate of 1 ^{O}C per every minute and kept in a -80 ^{O}C refrigerator until required.

2.2.2.2 Drug incubations

6 well plates were coated with rat tail collagen (Thermo Fisher Scientific, TX, USA) before the culture of the cells. Stock collagen was diluted with distilled water (Sigma-Aldrich, Dorset, UK) and pour into plates so that the final concentration of collagen in well plates was 100µg/µl. Plates were kept in centrifuge for 1 hr for coating, after which the diluted collagen was discarded and plates were washed with HBSS to remove all the remaining collagen floating in the plates. For drug incubations, 20,000 cells/well were cultured in collagen-coated 6 well plates. After two days of incubation in the incubator, the DMEM cell culture media was replaced by differentiating media containing 10mg/ml of insulin in all wells except the preadipocyte wells. The day of addition of insulin (start of differentiation process) was considered as day 0 of differentiation (Table 1). After 48 hours of differentiation, all media were discarded and replaced with fresh preadipocyte or differentiation media as the case may be. The differentiation media was replenished every 48 hours till day 10 when adipocytes were fully differentiated. Cells were treated with either vehicle (the solvent in which the drug was dissolved) or increasing doses of the drugs. Drug addition was conducted from day 2 to day 8 (total 4 drug additions) at every 48 hour intervals. This resembles the chronic toxicity model which was used to study chronic drug accumulation effects on various parameters. As metabolic toxic effects occur due to chronic exposure of drugs in humans so we used multiple drug exposure to cells to resemble our model more clinically. On day 10, supernatants and cell lysates were collected to measure adipokine levels and gene and protein expression respectively. Every experiment was repeated three times to check the reproducibility of the results and for statistical purposes.

CLO (1µM, 2µM, 20µM), OLA (0.2µM, 2µM, 20µM) and ARI (0.2µM, 1µM, 20µM) were used in the current study. The upper and lower concentrations of the therapeutic range were used for each drug. We also used an additional higher dose (20uM) to understand the effect of higher concentrations of AAPs which may be achieved as a result of accumulation within the adipose tissue. Different vehicles were used for each drug depending upon their solubility (CLO/LPV: methanol; OLA: DMSO; ARI: ethanol). Lopinavir (LPV) (20µM), an anti-HIV drug with known deleterious effects on adipocyte function was used as a comparator. All the drug's effect were compared with their respective vehicles.

Table 2.1 Drug treatment time line. Differentiating Medium (DM)

Preadipocytes (Preadipocytes media)		**Preadipocytes with DM**	**Differentiated Adipocytes (Differentiating media)**				
			Dose 1	**Dose 2**	**Dose 3**	**Dose 4**	**Collect lysates**
Day -2	**Day -1**	**Day 0**	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**

2.2.3 Measurement of Cell Viability

Cell viability was assessed in both preadipocytes and differentiated adipocytes by MTT assay. Preadipocytes were seeded in flat bottom 96-well plates at a density of 10,000 cells per well and incubated for 24 hr. A range of CLO, OLA and ARI concentrations (1-100µM) were added, with each concentration having 3 sets of quadruplicate wells and were incubated for 4 days. For differentiating adipocytes, cells were differentiated 48hrs after plating and incubated for further 24hr before adding

drugs. After 4 days all wells (both preadipocytes and differentiated adipocytes) received 20µl of MTT solution followed by incubation at 37°C for 2 hr. After 2 hrs, 100µl lysis buffer, prepared by adding 20% (w/v) SDS in dimethylformamide solution was added to all wells and incubated overnight. The absorbance of the plates was read at 595nm using a multimode detector (Beckman Coulter, CA, USA).

The MTT assay is based on the conversion of the tetrazolium salt MTT into a coloured and water-soluble formazan. The dehydrogenase enzymes of the viable cells reduce the yellow MTT solution to a purple colour at 37 °C. Therefore, the amount of formazan produced is directly proportional to the number of living cells providing a good indication of cell viability.

2.2.4 Lipid Accumulation

Two days after last drug addition, cells were washed with 2 ml/well of HBSS. Cells were then fixed in 2ml of formaldehyde (10% formaldehyde in HBSS) for 1 hr. After fixing, plates were washed with HBSS twice and 1.5ml filtered Oil Red O solution was added. Oil Red O stock solution was prepared by dissolving 0.5 g of Oil Red O powder in 100 ml of isopropanol. The working solution consisted of a mixture of 3 parts of Oil Red O stock solution and 2 parts of distilled water. The plates were placed on a shaker and left for 1 hr. Excess Oil Red O solution was then washed off with cold tap water and images were taken under a microscope. To quantify lipid accumulation, 1ml of 70 % isopropanol was added to each well to dissolve the lipid-bound Oil Red O stain and plates were put on a shaker for half an hour. 200µl isopropanol with stain was then transferred to a 96-well plate and absorbance was read using a multimode detector at 450 nm.

2.2.5 Measurement of secreted adipokines

2.2.5.1 Adiponectin

Adiponectin secretion was measured by solid phase sandwich ELISA kit. Supernatants were collected from the 6-well plates into Eppendorf tubes. Dilutions were prepared both for samples (1:1000) and standards. 100µl of diluted drug-treated samples and controls were added to the antibody coated 96-well plate. The plate was then covered and incubated for 1 hr at 37 ^{O}C. After 1 hr, solution was aspirated and all wells were washed 3 times with wash buffer. 100µl 1x detection antibody solution was added and incubated for 1 hr at 37 ^{O}C. After aspiration of the solution, all wells were then washed with wash buffer 3 times and 100µl 1x horseradish peroxidase-(HRP) was added to all wells. The plate was again incubated for 1 hr at 37 ^{O}C. After 1 hr, solution was aspirated and wells were washed 5 times with wash buffer. 100 µl substrate solution was added to all wells followed by an incubation period of 20min in the dark at room temperature. After the last incubation period, 100µl of stop solution was added to all of the wells and readings were made using a plate reader at 450nm. Adiponectin concentration in each sample was calculated as per manufacturer's instructions.

2.2.5.2 IL-6

100µl of standards and samples were added to the antibody-coated 96-well plate. The plate was covered with plate cover and incubated for 2 hr at room temperature. The solution was aspirated after incubation and wells were washed 4 times with wash buffer. 100µl of IL-6 biotin conjugate solution was added and incubated for 30 min at room temperature. After incubation, solution was aspirated and all wells were washed 4 times with wash buffer. Streptavidin-HRP (100µl) was added and 30min incubation was done at room temperature. After aspiration and washing wells, 4 times with wash buffer 100µl stabilised chromogen was added to each well. The plate was then allowed to incubate for 30min in dark at room temperature. In last step 100µl of stop solution

was added to all of the wells and readings were measured using a plate reader at 450nm. IL-6 concentration in each sample was calculated as per manufacturer's instructions.

2.2.5.3 TNF-α

After adding 100µl of standard and samples in appropriate well to the antibody-coated 96-well plate, 50µl of TNF-α biotin conjugate solution was added in all wells. Plate was covered and incubated for 90 min at room temperature. The solution was aspirated and wells were washed 4 times with wash buffer. 100µl of Streptavidin-HRP solution was added in each well and plate was incubated for 30 min at room temperature. After incubation solution was aspirated and wells were washed 4 times with wash buffer. After washing, 100µl of stabilised chromogen was added to each well and incubated for 30 min at room temperature in the dark. After last incubation 100µl of stop solution was added to each well and readings were measured using a plate reader at 450nm. TNF-α concentration in each sample was calculated as per manufacturer's instructions.

2.2.5.4 FFA

Cell culture supernatants were used to assess the FFA secretion by adipocytes. 50µl of standard dilutions and samples were added to 96 well plate followed by 2µl of Acyl-CoA Synthetase (ACS) reagent in each well. The plate was then incubated for 30 min at 37 ^{O}C. 50µl reaction mix (Table 2.2) was added to each well and incubated for 30min at 37 ^{O}C. The absorbance was then immediately measured using a plate reader at 570nm. FFA concentration in each sample was calculated as mentioned in the user manual.

Table 2.2 Reaction Mix for one reaction: FFA colorimetric assay

Component	Reaction Mix (µl)
Assay buffer	44
Fatty acid Probe	2
Enzyme Mix	2
Enhancer	2
Total	**50**

2.2.6 Isolation of mRNA

After aspirating the supernatant, cells were washed twice with HBSS. Cells were lysed by adding 500µl of Tri Reagent solution in each well. The lysates were transferred to autoclaved Eppendorf tubes. The lysates were mixed with 100µl of chloroform and the tubes were shaken vigorously for 15 seconds to get a homogenised mixture. The tubes were incubated at room temperature for 10 minutes. Afterwards, the tubes were centrifuged at 14000rpm for 15 minutes at 4 °C result in in the separation of a lower red (containing proteins), an interphase (containing DNA) and a colourless upper aqueous solution (containing RNA). The aqueous solution containing RNA was transferred carefully to a fresh tube and remaining solution was discarded. 25µl of isopropanol was added to each tube followed by brief mixing with vortex and incubation at room temperature for 10 minutes. After that, tubes were centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatants were then transferred to new tubes leaving any impurities at the bottom. 225µl of isopropanol was added to each tube followed by brief mixing with vortex and incubation at room temperature for 10 minutes. All tubes were centrifuged at 14000 rpm for 10 minutes at 4 °C. RNA precipitate in the form of white pellet at the bottom of the tube. The supernatant was discarded without disturbing pellet. 100µl of 75% ethanol was added and all the tubes

were centrifuged at 14000 rpm for 10 minutes at 4 °C. The supernatant was discarded without disturbing pellet. The RNA pellet was then air-dried for 10-15 minutes to remove all ethanol. 15µl of RNAase/DNAase free water was added and mixed gently by pipetting up and down. The quality and quantity of RNA extracted were checked using a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, TX, USA). All samples were stored at -20°C until used for reverse transcription.

2.2.7 Reverse transcription of total RNA

2µg of total RNA for each sample was added to reverse transcription reagents as shown in table 2.3.

Table 2.3. Components of reverse transcription reaction

Reagents	Quantity (µl)
Magnesium Chloride ($MgCl_2$)	5.5
Random Hexamer	1.25
dNTP Mixture	5
MultiScribe™ Reverse Transcriptase	0.875
Reverse Transcription Buffer	2.5
RNAase Inhibitor	0.5
Total	**15.625**

RNAase/DNAase free water was added to a tube containing total RNA and reverse transcription reagents making a total volume of 25µl in each tube. The tubes were mixed gently and then placed on a heating block of Veriti thermo-cycler from Applied Biosystems, Warrington, UK. The thermo-cycler was run according to reaction cycling parameter as shown in Table 2.4. On finishing reaction, the samples were mixed by vortex and centrifuged at maximum speed for few seconds. The cDNA

obtained was quantified using Nanodrop to check the quality and quantity of cDNA. This cDNA was further normalised to 20 ng/µl using RNAase/DNAase free water.

Table 2.4. Reaction cycling parameter in Thermal Cycler

Temperature	Time
25°C	**10 min**
48°C	**30 min**
95°C	**5 min**
4°C	**Indefinitely**

2.2.8 Gene expression by Real-time PCR

Gene expression was carried out using 7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA). TaqMan gene expression assays (Target gene - FAM labelled; Endogenous control -VIC labelled) and TaqMan gene expression master mix were used to perform duplex real-time PCR reactions to assess the levels of mRNA expression of target genes relative to mRNA levels of β-actin (housekeeping gene). Table 2.5 show the reaction components used. All reactions were performed in a 384-well plate (Thermo Fisher Scientific, TX, USA) using standard cycling parameters (Table 2.6). The mRNA expression was calculated using the comparative CT method according to the manufacturer's protocol. The fold expression of the gene of interest was expressed as 2^-(ΔΔCT).

Table 2. 5. Reaction components for Real-time PCR

Reagents	Quantity (µl)
TaqMan gene expression Assay (20x FAM dye-labelled)	1
TaqMan gene expression Assay (20x VIC dye-labelled)	1
TaqMan gene expression Master Mix	10
cDNA (1-100ng)	4
RNAase free water	4
Total	20

Table 2.6. Reaction cycling (40 cycles) parameter for Real-time PCR

Temperature	Time
50 °C	2 min
95 °C	10 min
95 °C	15 sec
60 °C	1 min

2.2.9 Protein extraction

Protein lysates were prepared using RIPA lysis buffer. After discarding the supernatant, cells were gently washed in ice-cold HBSS. 100µl RIPA buffer was added and a scraper was used to collect the cell lysates in Eppendorf tubes. Cells were then allowed to lyse on ice for 30min. After lysing, the Eppendorf tubes were centrifuged

to spin down insoluble materials for 30min at maximum speed at 4°C. The supernatant was collected containing the total cell protein.

2.2.10 Protein quantitation

Protein in lysates was quantitated by DC Protein Assay Kit. In this protocol, 20µl of reagent S was added to 1ml of reagent A which was considered as working reagent A. 5µl of protein standards and samples were added to the 96-well flat bottom plate. 25µl of working reagent A was added to all the wells followed by addition of 200µl of reagent B in all wells. The reagents were mixed by keeping the plate on a shaker for 1 min followed by 15 min incubation at room temperature. The readings were measured using a plate reader at 550nm. Protein concentration in each sample was calculated as mentioned in the user manual. 1-5 µg of protein was used for each sample for target protein expression.

2.2.11 Simple Western Assay

For quantitative analysis of protein expression, Simple Western System (WES, ProteinSimple, CA, USA) was used. Simple Western is a gel-free, blot free, film free and Western blot-like substitute technique. The WES machine performs size-based separation, immunoprobing, washing, detecting and analysis of the data (Chen et al., 2015). A duplex approach was applied in which both target protein antibody and endogenous control antibody were loaded in same wells. The biotinylated ladder was used for molecular weight determination. Protein lysates were mixed with 5x fluorescent master mix in a microcentrifuge tube to obtain final concentration between 0.2mg/ml to 0.8mg/ml. The samples and ladder were then denatured at 95 ^{O}C for 5 minutes. All the samples, ladder, primary and secondary antibodies, wash buffers and chemiluminescence substrate were loaded in the Simple WES plate in appropriate wells. The plate was centrifuged at 2500 rpm for 10 min. After that, the plate was

loaded in the WES machine for a period of 3 hrs. The resulting digital image was analysed using Compass software (Protein simple, CA, USA) as instructed in the user manual. The target protein was normalized against β-actin in the analysis. The following dilutions were used; PPARγ: 1: 25, lipin1: 1:50 and β-actin: 1:25.

2.3 Statistical Analysis

All experiments were done in triplicates. All statistical comparisons were made using unpaired t tests on Stat Direct software version 2.7.9. Differences were considered significant at $p \leq 0.05$.The result was shown as mean ± SD.

2.4 Results

2.4.1 Effect of AAPs on the cell viability of preadipocytes and differentiating adipocytes

2.4.1.1 Preadipocytes

None of the concentrations used for OLA and ARI showed any cell inhibition (Inhibitory concentrations (IC) 50= >100). However CLO showed cell inhibition only at higher concentrations (IC50=81.37μM) (Fig.2.1A). However all of our experiments were on differentiating adipocytes.

2.4.1.2 Differentiating Adipocytes

None of the concentrations used for OLA and ARI showed cell inhibition as IC50, is greater than 100 (IC50= >100). However CLO showed cell inhibition at higher concentrations (IC50= 60.57 μM). The maximum concentration we tested in subsequent experiments was 20uM, a concentration which had no appreciable cell death for any of the drugs tested (Fig.2.1B).

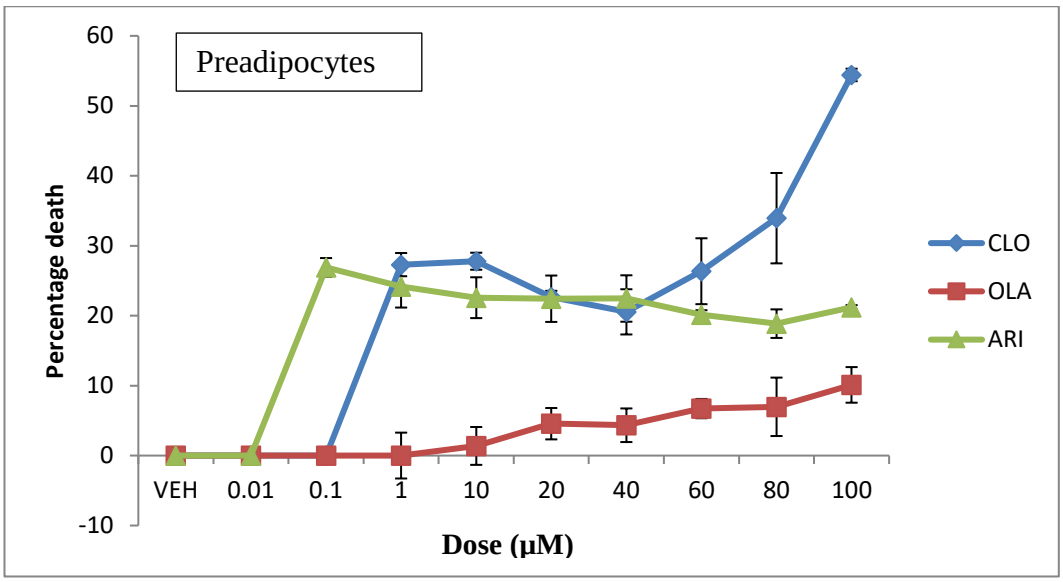


Figure 2.1 A. The percentage cell death caused by CLO, OLA and ARI (0.01-100 µM) on 3T3-F442A preadipocytes determined by MTT assay. Cells were treated with AAPs for 4 days followed by incubation with MTT solution for 2hr. Lysis buffer was added followed by overnight incubation. Absorbance was then measured by multimode detector. All experiments were done in triplicates. CLO: Clozapine; OLA: Olanzapine; ARI: Aripiprazole

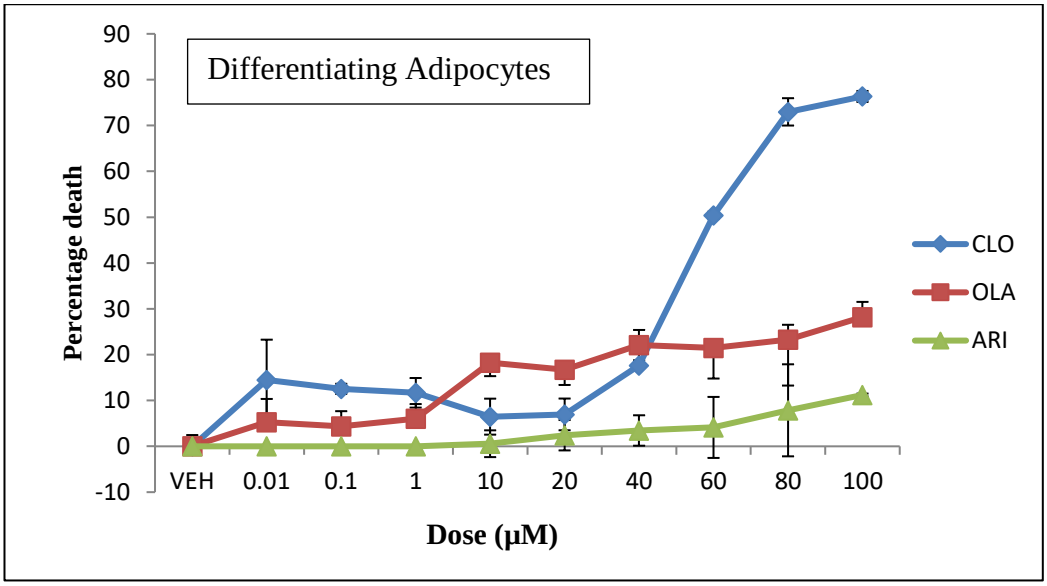


Figure 2.1 B. The percentage cell death caused by CLO, OLA and ARI (0.01-100 µM) on 3T3-F442A differentiating adipocytes determined by MTT assay. Cells were treated with AAPs for 4 days followed by incubation with MTT solution for 2hr. Lysis buffer was added followed by overnight incubation. Absorbance was then measured by multimode detector. All experiments were done in triplicates. CLO: Clozapine; OLA: Olanzapine; ARI: Aripiprazole

2.4.2 Effect of AAPs on lipid accumulation and adipogenesis

In differentiating adipocytes, a dose dependent significant increase in lipid accumulation was observed with CLO compared to vehicle-treated cells (1uM CLO: 1.22 absorbance units (AU) ±0.011; p=0.01, 2µM:1.35±0.017; p=0.001, 20µM:1.56±0.097; p=0.001, Vehicle: 1.05±0.06). OLA showed trend in increase in lipid droplets but this was not statistically significant (0.2µM:1.49±0.17; p=non-significant (ns), 2µM:1.53±0.15; p=ns, 20µM:1.57±0.14; p=ns). ARI, on the other hand did not show any change in lipid accumulation in any of the dose used. LPV, an anti-HIV drug which was used as comparator, showed significant reduction as compared to the vehicle as expected (0.86±0.043; p=0.01) (Fig. 2.2 A). This was also evident from the photomicrographs taken following Oil Red O staining (Fig 2.2 B).

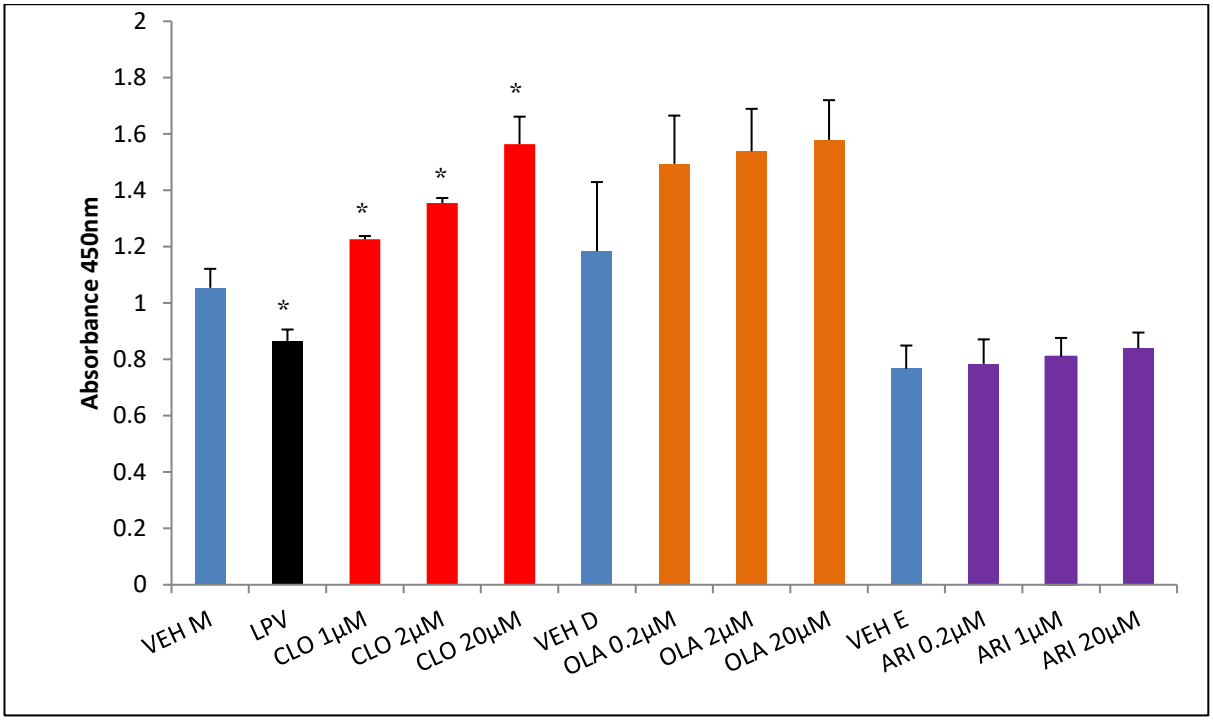


Figure 2.2.A. Quantitation of lipid bound stain. 1ml of 70 % isopropanol was added to each well to dissolve the lipid-bound Oil Red O stain and plates were put on a shaker for half an hour. 200µl isopropanol with stain was then transferred to a 96-well plate and absorbance was read using a multimode detector. Graph showing effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in triplicates. Data was shown as Mean ±SD; p≤0.05. * VEH M vs LPV/CLO. VEH M Vehicle. Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

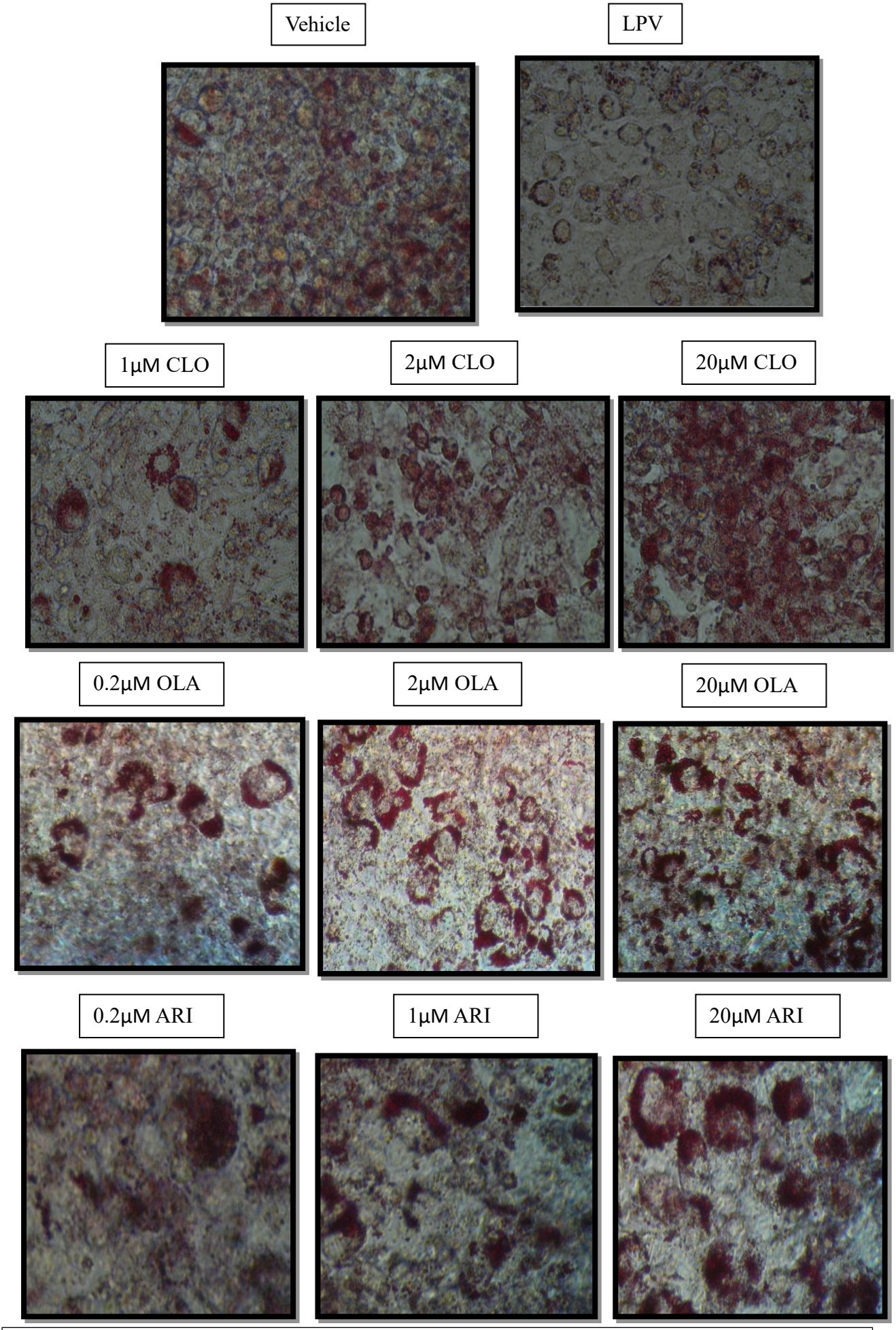


Figure 2.2 B. Photomicrographs (20x magnification) showing Oil Red O staining of 3T3-F442A adipocytes on 10th day post differentiation treated with various concentration of CLO, OLA and ARI. CLO: Clozapine OLA: Olanzapine, ARI: Aripiprazole: LPV: Lopinavir

2.4.3 Effect of AAPs on adipocytokine secretions

2.4.3.1 Adiponectin secretion

Treatment with CLO showed dose dependent significant increase in adiponectin secretion as compared to vehicle (1µM:555.28ng/ml±51.70; p=0.005, 2µM:661.07±69.20; p=0.002, 20µM:736.77±66.06; p=0.001, vehicle: 323.16±53.07). However both OLA (2µM: 1115.82±16.26; p=0.003, 20µM:990.70±6.08; p=0.0002, vehicle: 1243.51±31.03) and ARI (1µM:347.73±7.74; p=0.001, 20µM:322.40±4.31; p=0.0003, vehicle: 408.59±12.07) showed significant reduction in adiponectin secretion. LPV showed significant reduction in adiponectin secretion compared to vehicle as expected (114.52± 42.52; p=0.006) (Fig 2.3 A).

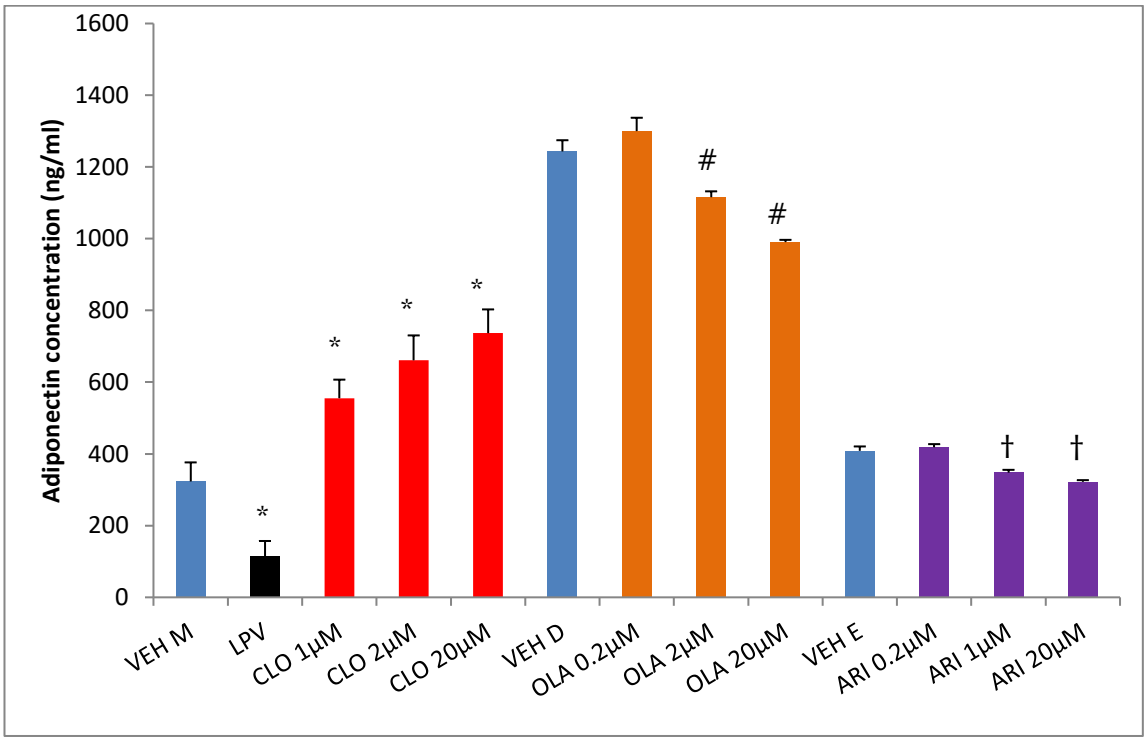


Figure 2.3 A. Effect of AAPs on Adiponectin secretion. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. Supernatants were collected on day 10 and adiponectin secretion was measured by solid phase sandwich ELISA as described by manufacturer's instructions. All experiments were done in triplicates. Data represent mean ± SD; $p \leq 0.05$. * VEH M vs LPV/CLO, # VEH D vs OLA, † VEH E vs ARI. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

2.4.3.2 IL-6 secretion

None of the concentrations used for CLO (1uM: 19.30±3.91; p=ns, 2µM:19.73±3.61; p=ns, 20µM:22.84±3.96; p=ns, Vehicle: 21.03±3.35) and OLA (0.2uM: 24.80±1.24; p=ns, 2µM:24.88±0.80; p=ns, 20µM:24.97±1.93; p=ns, Vehicle: 22.26±0.40) showed any change in secreted IL-6. ARI showed increase in IL-6 secretion only at the higher dose (20µM:74.49±2.44; p=0.0004, Vehicle: 12.28±0.41). LPV showed significant increase in IL-6 secretion as expected (75.59± 3.35; p=0.0002) (Fig 2.3B).

2.4.3.3 TNF-α secretion

CLO showed dose dependent significant increase in TNF-α secretion compared to vehicle (1uM: 37.10±0.16; p=0.0004, 2µM:43.17±0.34; p=<0.0001, 20µM:45.24±0.16; p=<0.0001, Vehicle: 32.89 ±0.34). However none of the doses of OLA (0.2uM: 21.33±0.29; p=ns, 2µM:21.12±0.30; p=ns, 20µM:20.95±0.31; p=ns, Vehicle: 21.69 ±0.45) and ARI (0.2uM:21.67±0.12; p=ns, 1µM:21.43±0.10; p=ns, 20µM:21.28±0.08; p=ns, Vehicle: 21.91±0.09) showed any change in secretion while LPV caused increase in TNF-α secretion (42.85± 0.37; p=<0.0001) (Fig 2.3C).

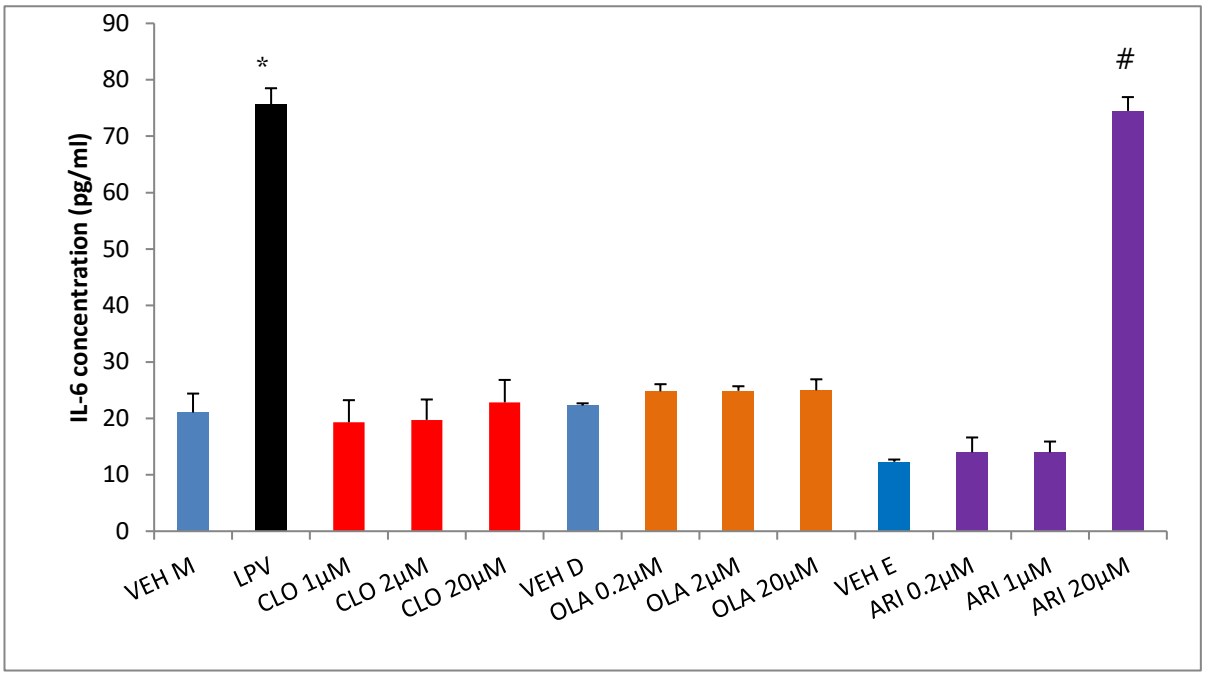


Figure 2.3B. Effect of AAPs on IL-6 secretion. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. Supernatants were collected on day 10 and IL-6 secretion was measured by solid phase sandwich ELISA as described by manufacturer's instructions.. All experiments were done in triplicates. Data represent mean ± SD; $p \leq 0.05$ *VEH M vs LPV, # VEH E vs ARI. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

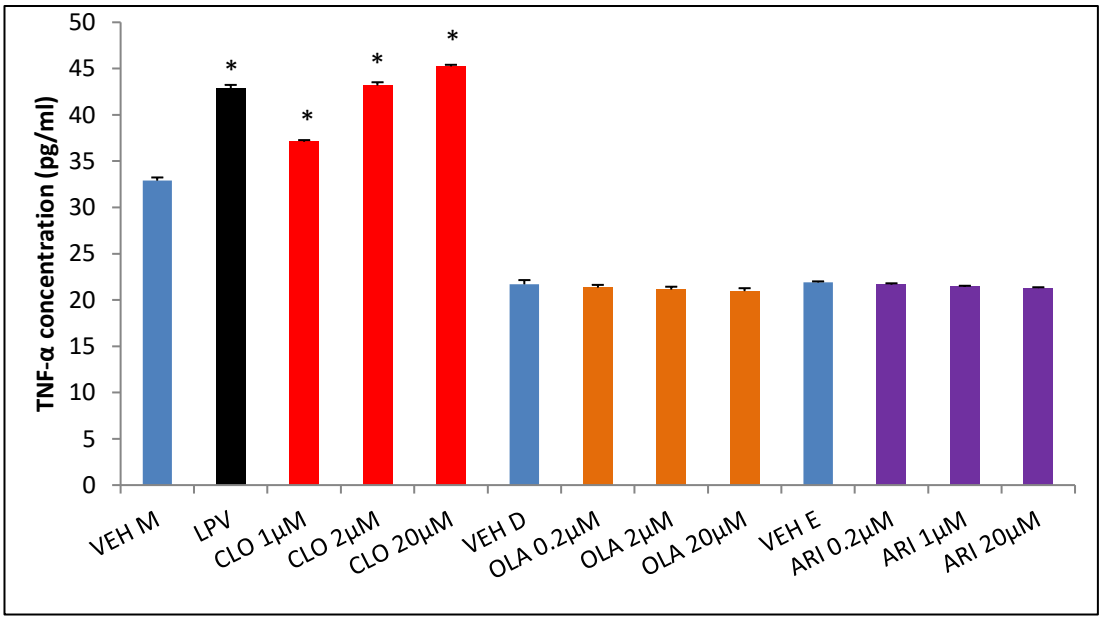


Figure 2.3C. Effect of AAPs on TNF-α secretion. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. Supernatants were collected on day 10 and TNF-α secretion was measured by solid phase sandwich ELISA as described by manufacturer's instructions. All experiments were done in triplicates. Data represent mean ± SD; $p \leq 0.05$ *VEH M vs LPV/CLO. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

2.4.3.4 Free Fatty Acid Secretion

FFA was significantly and dose dependently increased by CLO as compared to the vehicle (1uM: 0.119±0.001; p=0.0003, 2µM:0.124±0.002; p=0.001, 20µM:0.131±0.004; p=0.007, Vehicle: 0.105 ±0.0009). However OLA increased FFA secretion only at therapeutic concentration (0.2µM:0.114±0.003; p=<0.0001, Vehicle: 0.100±0.002) and not at other concentrations (2µM:0.103±0.004; p=ns, 20µM:0.099±0.002; p=ns). On the other hand, ARI did not show any change in FFA at therapeutic doses (0.2µM:0.113±0.0005; p=ns, 1µM:0.117±0.0009; p=ns) but increased at the higher dose (20µM: 0.137 ±0.001, p=0.0002, Vehicle: 0.115±0.0007). LPV, as expected, showed a significant increase in FFA secretion (0.116±0.0007; p=<0.0001) (Fig 2.3 D).

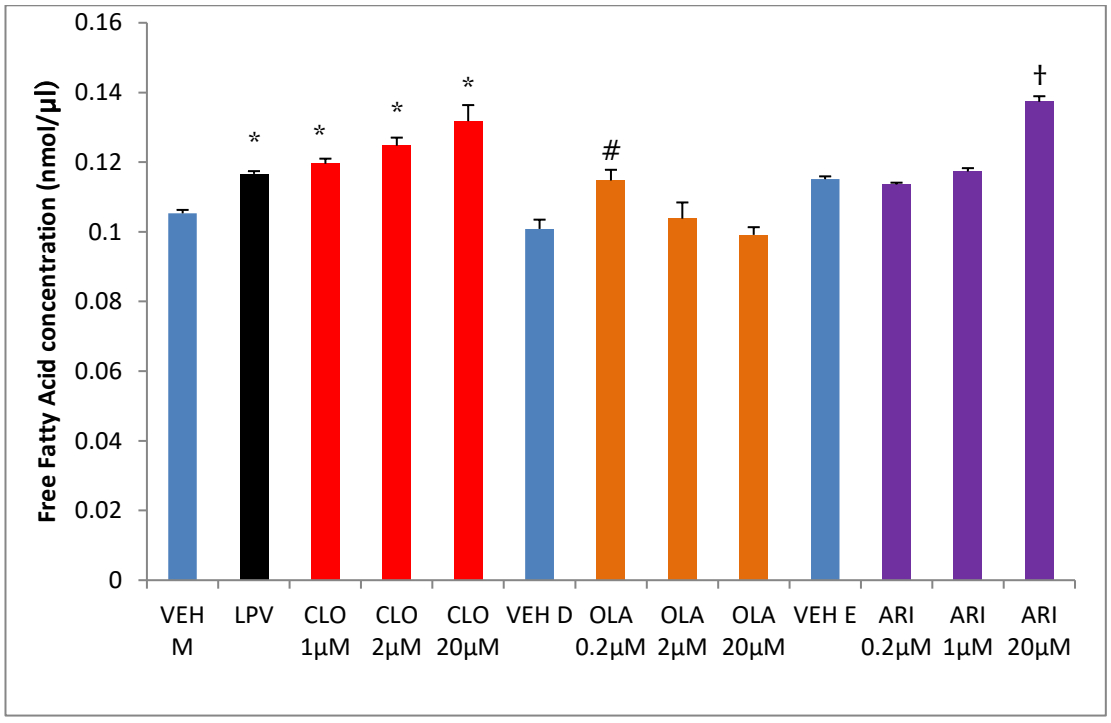


Figure 2.3D. Effect of AAPs on FFA secretion. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. Supernatants were collected on day 10 and FFA secretion was measured by colorimetric method as described by manufacturer's instructions. All experiments were done in triplicates. Data represent mean ± SD; $p \leq 0.05$ *VEH M vs LPV/CLO, # VEH D vs OLA, † VEH E vs ARI. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

2.4.4 Effect of AAPs on Adipogenic gene and protein expression

2.4.4.1 Adiponectin mRNA expression

Adiponectin (*adipoQ*) gene showed a significant dose dependent increase in expression following treatment with CLO (1uM: 1.69±0.17; p=0.03, 2µM:2.06±0.07; p=0.006, 20µM:2.62±0.35; p=<0.0001). However none of the OLA (0.2uM: 1.07±0.04; p=ns, 2µM:0.99±0.03; p=ns, 20µM:0.93±0.03; p=ns) and ARI (0.2uM: 0.94±0.04; p=ns, 1µM:0.99±0.05; p=ns, 20µM:1.03±0.06; p=ns) concentrations tested showed any change in adiponectin expression. LPV showed significant decrease in adiponectin gene expression as expected (0.69±0.14; p=0.04) (Fig 2.4 A).

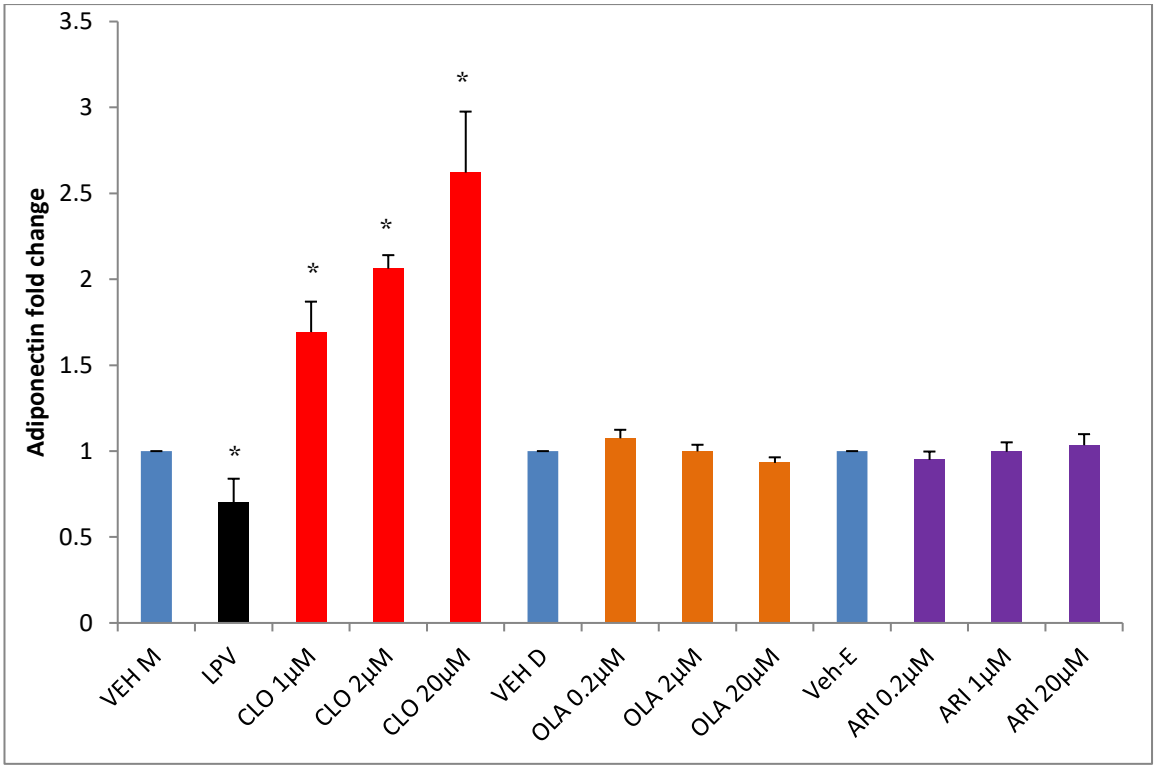


Figure 2.4A. Effect of AAPs on *adipoQ* mRNA expression. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. RNA extracted and *adipoQ* mRNA was assessed using RT-PCR. β-actin was used as endogenous control. All experiments were done in triplicates. Data represent mean fold change ± SD; $p \leq 0.05$ * VEH M vs LPV/CLO. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

2.4.4.2 ppar-γ mRNA expression

After treatment with CLO and ARI, *ppar-γ* showed significant dose dependent (2μM CLO; 2.79±0.43; p<0.0001, 20 μM CLO: 3.58±0.38; p<0.0001, 1μM ARI: 1.55±0.10; p=0.03, 20 μM ARI:1.87 ±0.15,p=0.0003) increase in mRNA expression as compared to vehicle while OLA showed an increase in expression only at 0.2μM (1.08±0.01, p=0.04). LPV (0.78±0.07,p=0.04) showed significant decrease in *ppar-γ* expression compared to the vehicle (Fig 2.4B).

2.4.4.3 PPARγ protein expression

Two concentrations (therapeutic and higher dose) for each of the drug was used to investigate drugs effect on PPARγ and lipin1 (LPIN1) protein expression.

CLO treatment showed dose dependent (1μM:176.53±7.84; p=0.002, 20μM:307.34±26.30; p=0.0001) significant increase in PPARγ expression as compared to the vehicle while OLA did not show any change (0.2μM:105.38±2.54; p=ns, 20μM:90.22±3.56; p=ns). Treatment with ARI showed a significant increase in PPARγ expression only at the highest dose used (0.2μM: 85.15±7.33; p=ns, 20μ M: 261.68±48.46, p=0.02).LPV showed trend to decrease but the result was non-significant (59.95±27.64, p=ns) (Fig 2.4C).

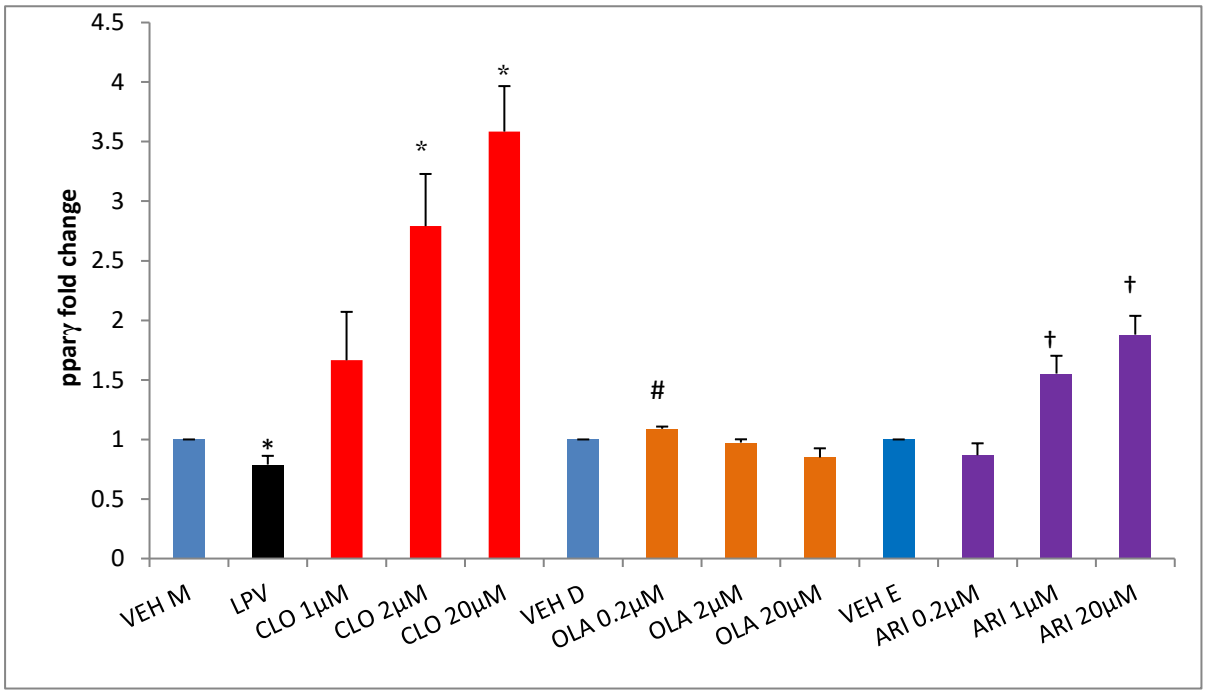


Figure 2.4B. Effect of AAPs on *pparγ* mRNA expression. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10days. RNA extracted and *pparγ* mRNA was assessed using RT-PCR. β actin was used as endogenous control. All experiments were done in triplicates. Data represent mean fold change ± SD; $p \leq 0.05$ * VEH M vs CLO, #VEH D vs OLA, † VEH-E vs ARI. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole

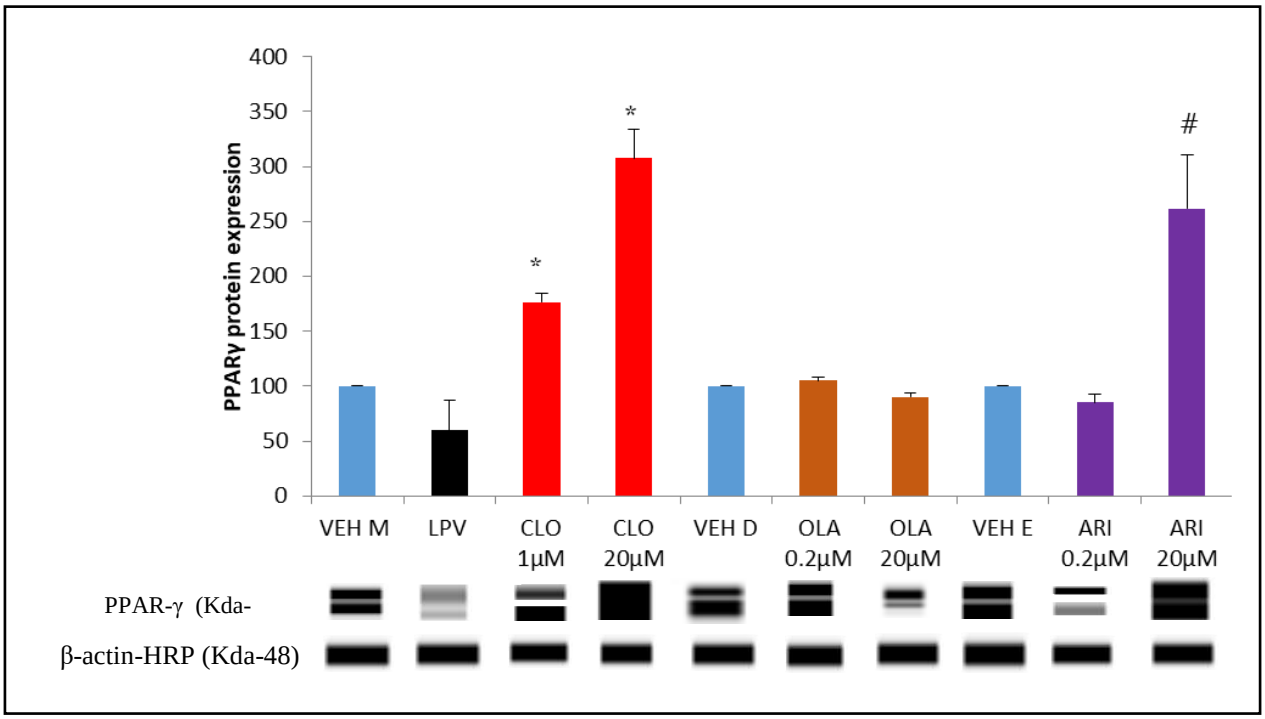


Figure 2.4C. Effect of AAPs on PPARγ protein expression. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. Protein isolated and PPARγ expression was assessed by western blotting. β-actin was used as endogenous control. Data represent mean ± SD; $p \leq 0.05$ *VEH M vs CLO, # VEH E vs ARI. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

2.4.4.4. Lipin1 mRNA expression

CLO treatment showed significant increase in *lpin1* mRNA expression (2µM:1.66±0.08, p=0.005, 20µM:1.94±0.10, p=0.004) as compared to vehicle. OLA caused decrease in expression only at higher dose (20µM:0.92±0.03, p=0.05) while ARI showed decrease only at therapeutic doses (0.2µM:0.78±0.02, p=0.004, 1µM:0.88±0.03, p=0.004). LPV showed significant decreased as compared to vehicle (0.64± 0.11, p=0.03) (Fig.2.4D).

2.4.4.5 Lipin1 protein expression

CLO treatment showed significant increase in LPIN1 expression only at higher dose (20µM:213.46±26.43, p=0.02) compared to vehicle while both OLA (0.2µM:110.02±9.38, p=ns, 20µM:92.44±14.62,p=ns) and ARI (0.2µM:94.62±20.51,p=ns, 20µM:107.08±31.07,p=ns) did not show any change in any of the concentration used. Treatment with LPV showed significant decreased (72.38±9.00, p=0.003), as compared to vehicle (Fig 2.4E).

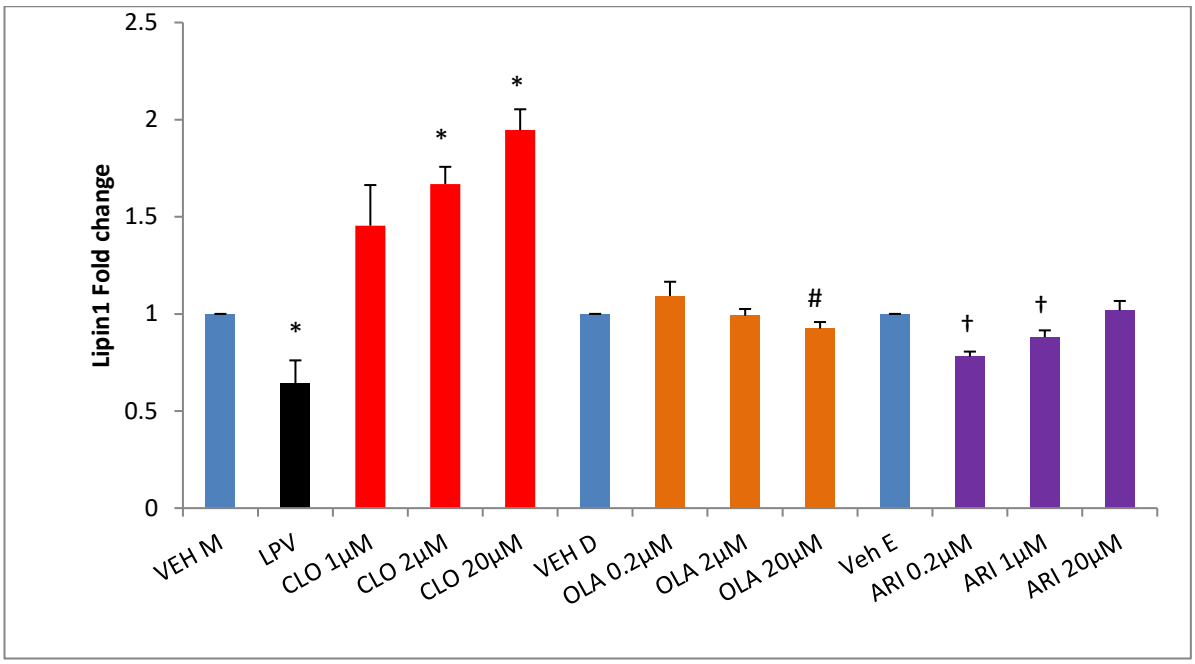


Figure 2.4D. Effect of AAPs on *lipin1* mRNA expression. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10days. RNA extracted and *lipin1* mRNA was assessed using RT-PCR. β-actin was used as endogenous control. All experiments were done in triplicates. Data represent mean fold change ± SD; $p \leq 0.05$ * VEH M vs CLO, #VEH D vs OLA, † VEH-E vs ARI. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

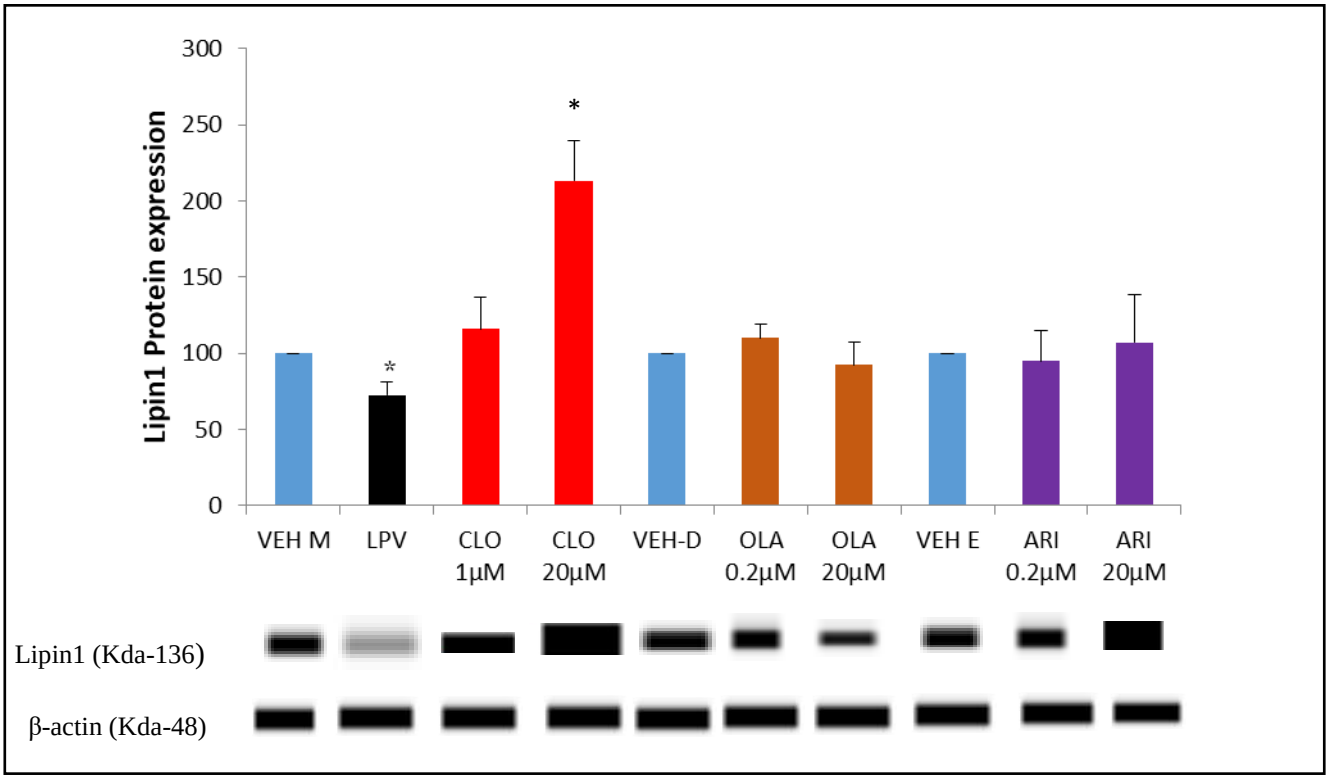


Figure 2.4E. Effect of AAPs on Lipin1 protein expression. Differentiated adipocytes were treated with increasing doses of CLO, OLA and ARI over a period of 10 days. Protein isolated and Lipin1 expression was assessed by western blotting. β-actin was used as endogenous control. Data represent mean ± SD; $p \leq 0.05$ *VEH M vs LPV/CLO. AAPs: Atypical antipsychotics, VEH M: Vehicle Methanol, VEH E: Vehicle Ethanol, VEH D: Vehicle DMSO, LPV: Lopinavir, CLO: Clozapine, OLA: Olanzapine, ARI: Aripiprazole.

2.5 Discussion

AAPs are the drug of choice for treatment of schizophrenia due to less risk of extrapyramidal side effects. However, it causes metabolic adverse effects such as weight gain, insulin resistance, and dyslipidemia. Various mechanisms ranging from central receptor effects to the peripheral inflammatory, oxidative stress, insulin resistance, and lipid accumulation has been proposed but none of them have been declared as an absolute mechanism (Hu et al., 2010). We used 3T3-F442A preadipocyte cell line as a murine *in vitro* model to characterise the underlying mechanism. We preferred to use this cell line model compared to other 3T3, such as 3T3-L1, OP9 or C3H10T1/2 as 3T3-F442A has more ability to accumulate fat and showed more advanced commitment towards adipocyte differentiation (Ruiz-Ojeda et al., 2016). Schizophrenic patients took AAPs for long period of time leading to accumulation of drugs in the body which cause metabolic adverse effects. To mimic this condition, an *in vitro* chronic toxicity model approach was applied in which drugs were given multiple times, to study the effect of drugs on lipid accumulation and various adipogenic parameters. Previous studies have proposed increased lipid accumulation and increased adipogenesis as a mechanism of weight gain leading to insulin resistance and metabolic syndrome caused by AAPs (Vestri et al., 2007, Yang et al., 2007, Hemmrich et al., 2011). Our result showed an increase in lipid accumulation by CLO and OLA but not ARI. This is consistent with Hu and colleagues study which showed an increase in adipogenesis by CLO (Hu et al., 2010). Our result is also consistent with recently published data reported dose-dependent increase by CLO in lipid droplets using 3T3-L1 as a model (Tsubai et al., 2017). Our results also coincide with Hemmrich and colleagues research (Hemmrich et al., 2011) which showed increased lipid accumulation by CLO while OLA marginally increases it. Hemmrich study also showed a decrease in lipid accumulation by ARI which was not observed in our study. Our data clearly depicted that CLO and OLA cause

accumulation of excess body fat compared to ARI. This showed CLO and to some extent OLA, poses a potential risk to cause weight gain, obesity and metabolic syndrome. Our study showed a comparison in terms of lipid accumulation among AAPs, which was not showed in previous studies, which clearly showed role of CLO and OLA in causing weight gain, obesity and metabolic adverse effects. This also links to published clinical data, span over 20 years on psychiatric schizophrenic patients treated with AAPs, which reported 52.3 percent patients to be associated with weight gain after using CLO and OLA compared to ARI treated group (Francesco and Cervone, 2014). Obesity is characterised by increased size and accumulation of lipid droplets so we can suggest from our data that CLO and OLA directly affect the cells and increased adipogenesis leading to deposition of excess fat, which might be one of the mechanisms by which AAPs cause weight gain. However, there are also contradictory results related to the effect of AAPs on adipogenesis. A study by Sertie and colleagues showed that lipid droplet accumulation did not change significantly even at the higher dose of CLO (30μM) and OLA (100μM) (Sertie et al., 2011). Also, Hauner and colleagues reported no direct effect of CLO on fat cell formation (Hauner et al., 2003) but in their study, CLO was given only for 24hr which might not show effect while our study spans around 10 days with multiple drug additions leading to chromic accumulation of drugs which produced its deleterious effects by increasing adipogenesis and lipid accumulation.

Adipogenesis triggers important transcriptional changes in adipocytes without which adipocytes cannot be mature. Most important is the increased expression of PPARγ, considered as a master regulator of adipogenesis. Previous studies showed an increase in PPARγ mRNA expression by CLO (Sertie et al., 2011, Sarvari et al., 2014) while OLA did not show any change (Sarvari et al., 2014, Hu et al., 2010, Sertie et al., 2011). Our study is consistent with these findings. Our results also showed no effect

on PPARγ mRNA expression by therapeutic concentrations of ARI which coincides with Sarvari and colleagues' study (Sarvari et al., 2014). These previous results and our results showed the importance of transcriptional factor such as PPARγ in adipogenesis without which adipocytes cannot be mature and perform physiological and molecular functions. It is quite possible that AAPs, increased adipogenesis by activating PPARγ which caused increased formation of lipid droplets and lipid accumulation leading to weight gain. However, there are studies which contradict with these results. Nimura et al reported no change in PPARγ and adiponectin gene by the higher dose of OLA (Nimura et al., 2015). Another study by Brandl et al on 216 schizophrenic patients who received antipsychotics for 14 weeks, showed that neither PPARγ nor adiponectin gene variants had any association with antipsychotic-induced weight gain (Brandl et al., 2014b). In the current study, CLO but not OLA or ARI showed an increase in lipin1 gene and protein expression. Hence, there is a possibility that AAPs such as CLO cause metabolic adverse effects by affecting adipogenesis through lipin1 by increasing adipogenesis, leading to weight gain and obesity. This is the first study to investigate the expression of lipin1 when treated with AAPs; our results showed increase in lipin1 expression by CLO while OLA and ARI did not show any noticeable effect. Lipin1 worked as co-activator of PPARγ2 and regulates the network between PPARγ2 and C/EBPα activating the genes required for adipogenesis. Studies showed that lack of lipin1 gene in mice, resulted in failure to develop mature adipocytes leading to lipodystrophy and fatty liver while transgenic mice with increased lipin1 expression resulted in hypertrophic adipocytes (Koh et al., 2008, Reue and Zhang, 2008, van Harmelen et al., 2007).This suggest that lipin1 can be potential transcriptional factor, which might be affected by AAP resulted in lipid accumulation, obesity and weight gain. However in human, the relationship of lipin1 expression with obesity and insulin sensitivity is complex. Various studies have shown the

downregulation of lipin1 gene in obese and diabetes mellitus 2 subjects (van Harmelen et al., 2007, Yao-Borengasser et al., 2006) while other studies showed no correlation between lipin1 and insulin sensitivity (Miranda et al., 2010). The mechanism of decreased lipin1 expression in obese patients is still unknown. Future studies are required to characterise the role of lipin1 in AAPs induced metabolic adverse effects.

Lower adiponectin levels have been associated with insulin resistance and diabetes mellitus (Richards et al., 2006). Many previous clinical studies have shown decreased adiponectin secretion in patients treated with AAPs (Klemettila et al., 2014, Hanssens et al., 2008). Our results show increase in adiponectin secretion by CLO which contradict with recently published data showing a decrease in adiponectin secretion by CLO (Tsubai et al., 2017). Our study also contradicts with the clinical results which reported a decrease in adiponectin secretion by CLO and OLA (Wampers et al., 2012, Sugai et al., 2012, Klemettila et al., 2014, Hanssens et al., 2008). We performed CLO accumulation assay (explained in chapter 3) to find the cause of discrepancy between our model and other published data. It was found that less CLO was accumulated inside the 3T3-F442A adipocytes compared to control which might play role in contradictory data of adiponectin secretion. This experiment suggest involvement of adipocyte membrane transporter or receptor which block the entry of drugs into the cells however more research is required to find the exact cause.

Another factor associated with antipsychotic-induced metabolic toxicity is the role of inflammation (Sarvari et al., 2014). The inflammatory markers like IL-6 and TNF-α can act on insulin receptor and block its phosphorylation or might affect fatty acid metabolism which leads to insulin resistance (Makki et al., 2013). Our result did not show any change in IL-6 secretion by CLO and OLA however, ARI showed an increase but only at a higher dose. Our *in vitro* results contradict with published clinical data that showed an increase in IL-6 levels after CLO treatment (Leonard et

al., 2012, Klemettila et al., 2014). However it should be noted that clinical studies showed an inconsistent result with OLA; Hori et al (Hori et al., 2007) showed no effect while Kluge et al (Kluge et al., 2009) showed a decrease in IL-6 levels. Sobis and colleagues' research showed decrease IL-6 secretion by ARI which also contradict with our results (Sobis et al., 2015). The contradictory result showed by our model might be associated with decreased accumulation of CLO inside the cell as mentioned earlier. Also our study span around 10 days which might not produce the required effect, due to short duration of study in which drug was not accumulated enough to produce effect, compared to schizophrenic patients taking AAPs for longer period of time .

In the context of TNF-α levels, our result correlates with Sarvari and colleagues study in terms of only OLA and ARI which did not show any change. However, our data showed increased TNF-α levels by CLO which contradict with Sarvari et al study showing no change (Sarvari et al., 2014). Clinically, TNF-α level was increased by CLO and OLA (Pollmacher et al., 2000, Kluge et al., 2009) and decreased by ARI (Sobis et al., 2015). However, our data correlate clinically only with CLO, suggesting its potential is causing metabolic adverse effects. TNF-α could be a potential factor to study as it has been reported to be associated with insulin resistance (Guilherme et al., 2008). It has been proposed that TNF-α suppress insulin signalling leading to insulin resistance (Guilherme et al., 2008). Changes in TNF-α levels by AAP especially CLO suggests that these drugs induced inflammation in adipose tissue activating a cascade of factors and events which not only affect physiological functions of adipose tissue but also affect other organs leading to insulin resistance.

FFA also play an important role in metabolic syndrome. Insulin creates a balance between lipogenesis and lipolysis however during insulin resistance there is an increase lipolysis which increases the release of FFA. This FFA is either stored in the

adipose tissue leading to obesity or goes to the liver causing increased production of triglycerides resulting in hepatic steatosis. FFA also cause glucose intolerance by decreasing glucose intake by the muscle leading to diabetes (Adiels et al., 2005). Previous studies showed increased levels of FFA by CLO whereas OLA decreased the levels and ARI cause no change (Kaddurah-Daouk et al., 2007, Canfran-Duque et al., 2013, Jassim et al., 2012). Our study coincides with published CLO and ARI data except OLA which showed decreased levels in other studies while our study did not show any change in FFA levels (Jassim et al., 2012, Kaddurah-Daouk et al., 2007). Our data showed role of AAP especially CLO in causing lipolysis releasing excess FFA which then stored in peripheral tissues, disturbing their physiological functions either causing steatosis or glucose intolerance which ultimately lead to insulin resistance. It is possible that increase in FFA levels might be one of the mechanisms causing metabolic adverse effects, however, more studies required to characterise its role in metabolic syndrome.

In summary, the data presented here correlates with some of the published literature, but also contradicts with some studies. Of the AAPs studied, CLO caused the greatest amount of metabolic toxicity. Contrary to expectations and the published literature, it caused an increase in adiponectin levels. In next chapter, we have used another model (primary human adipocytes) to validate the murine data but also to explain the discrepant results we obtained with the murine cells.

Chapter 3

Effect of Atypical antipsychotics on adipogenesis using *in vitro* primary human adipocytes

3.1 Introduction

There are three types of adipose tissue in the human body; white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipose tissue. Beige adipocytes reside in WAT and on stimulation, acquire a thermogenic phenotype similar to brown adipocytes (Kiefer, 2017). The human body has abundant WAT which consist of mostly white adipocytes with a single large cytoplasmic lipid droplet and a nucleus which is located to the side of the cell. Along with white adipocytes, adipose tissue also contains immune cells, fibroblasts, endothelial cells and neuronal cells (Cristancho and Lazar, 2011). Adipogenesis comprises of a complex process involving the determination of preadipocytes from stem cells, and their terminal differentiation into mature adipocytes. Adipogenesis is mediated by several transcription factors which play an important role in the maturation of adipocytes. The most important regulator of adipogenesis is considered to be PPARγ. There are 2 isoforms of PPARγ, 1 and 2, which exist due to alternative splicing. PPARγ2 is mainly expressed in the adipose tissue. It has been found that overexpression of PPARγ induces adipocyte differentiation and adipogenesis while cells lacking PPARγ resulted in failure of the cells to become mature adipocytes. PPARγ also induce expression of genes, LPIN1, SREBP, GLUT 4, ADIPOQ and LPL (lipoprotein lipase), all of which play important role in lipogenesis and glucose metabolism (Rosen and MacDougald, 2006, Tontonoz and Spiegelman, 2008). It has been reported that subcutaneous adipose tissue which has the highest lipid accumulation capability showed the highest expression of PPARγ and C/EBP-α compared to abdominal mesenteric and omental adipose tissue (Tchkonia et al., 2002). The previous study has reported the connection between PPARγ and lipin1 proposing that it reinforces the interaction between C/EBPα and

PPARγ2 thus increase the production of adipocyte-specific genes necessary for maturation and maintenance of adipocytes (Figure 3.1) (Reue and Zhang, 2008).

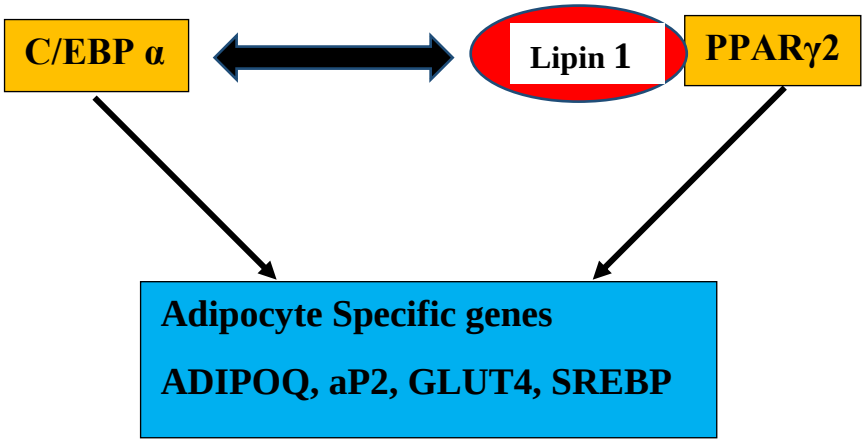


Figure 3.1. Interaction between Lipin1 and PPARγ

Leptin and adiponectin are considered to be the two most important hormones released by WAT. Leptin suppresses satiety factor that suppresses food intake. There is a positive correlation between plasma levels of leptin and WAT mass (Potvin et al., 2015). Adiponectin is considered to be an important biomarker for indicating insulin sensitivity. There has been an inverse relationship between adiponectin levels and WAT mass. It also secretes proinflammatory factors, important ones are IL-6 and TNF-α (Pollmacher et al., 2000).

AAPs are considered to be the gold standard for schizophrenia; however, these drugs result in metabolic adverse effects which include weight gain, insulin resistance, glucose intolerance and dyslipidemia, all of which are risk factors for metabolic complications such as obesity, diabetes mellitus, and cardiovascular disease. Various clinical trials have showed that among various AAPs prescribed, CLO and OLA are considered to be the worst in causing metabolic adverse effects; QTP, RISP and AMI are moderately implicated; and, ARI and ZIP are the lowest in causing metabolic adverse effects (Tandon et al., 2008, Naber and Lambert, 2009). Various mechanisms have been proposed for AAP-induced metabolic toxicity. According to various *in vitro*

studies (primary human and rat adipocytes) (Himmerich et al., 2011, Boyda et al., 2010, Nimura et al., 2015, Pavan et al., 2010, Loffler et al., 2016) and clinical studies (Haupt, 2006, Haddad, 2005, Swartz et al., 2008, Kahn et al., 2008, Francesco and Cervone, 2014), lipid accumulation leading to weight gain and obesity is considered to be one factor causing metabolic adverse effects. It has been shown that hypertrophy and hyperplasia of adipocytes resulted in the accumulation of the fats. This was considered to be the main pathway leading to lipid accumulation and obesity (Landgraf et al., 2015). However, there is also evidence of metabolic disturbances by CLO without weight gain. It was shown that CLO cause diabetes, dyslipidemia and impaired glucose tolerance in the absence of weight gain (Steffenhagen et al., 2012, Zimmermann et al., 2003). Another mechanism proposed is the direct effect of AAPs on insulin action causing its impairment which badly affects glucose transport and balance between lipogenesis and lipolysis (Vestri et al., 2007).Various clinical trials in schizophrenic patients taking CLO and OLA also showed a decrease in adiponectin levels which depicts the relationship of decreased adiponectin levels with insulin resistance proposing its mechanistic role in metabolic toxicity (Lu et al., 2015, Bartoli et al., 2015b, Swartz et al., 2008). Leptin not only decreases appetite leading to weight loss but also involved in regulation of glucose metabolism and immune system. Various clinical studies showed a mixed result with some studies showing an increase in leptin levels (Potvin et al., 2015) by AAPs whereas others showed no change (Tanaka et al., 2008). It was suggested that leptin resistance might be the reason causing weight gain in schizophrenic patients taking AAPs (Caro et al., 1996, Potvin et al., 2015). Inflammation might be involved in causing metabolic toxicity by AAPs. It was suggested that pro-inflammatory marker levels especially IL-6 and TNF-α were increased in schizophrenic patients taking AAPs. These markers blocked IRS1 phosphorylation resulting in insulin resistance (Hotamisligil et al., 1995, Makki et al.,

2013). The other proposed mechanism was related to TNF-α which act on adipocytes and cause increase in lipolysis and decrease triglyceride synthesis. This resulted in increasing levels of FFA in the plasma. These FFA cause accumulation of triglycerides and other activated lipids in peripheral tissues like liver, pancreas and beta cells. This accumulation disrupts the normal metabolic function of these tissues leading to insulin resistance (Guilherme et al., 2008). Previous studies showed an increase in IL-6 and TNF-α levels in schizophrenic patients taking AAPs (Mori et al., 2015, Klemettila et al., 2014, Victoriano et al., 2010, Pollmacher et al., 2000). Other proposed mechanisms by which AAPs caused metabolic toxicity include, effects on neuroreceptors (serotonin, dopamine, histamine) leading to weight gain (Balt et al., 2011, Masaki et al., 2004, Reynolds and Kirk, 2010), impairment of mitochondrial functions (Ji et al., 2009) and production of reactive oxygen species (Miljevic et al., 2013).

The absolute mechanism causing AAPs-induced metabolic adverse effects is unknown. This chapter investigate the effect of AAPs on adipogenesis using primary human adipocytes as a model and attempt validation of the results obtained in Chapter 2 using a murine 3T3-F442A model.

3.1.1 Rationale for the study

Several mechanisms have been proposed which are thought to be mechanistically involved in metabolic adverse effects caused by AAPs so far. Among AAPs, CLO and OLA has been suggested to cause metabolic adverse effects compared to other AAPs (Nasrallah, 2008). This could be due to their effects on adipogenesis (Loffler et al., 2016, Francesco and Cervone, 2014); or changes in their transcription factors (Sertie et al., 2011); or inflammatory cytokines (Guilherme et al., 2008); or adipokines such as adiponectin and leptin (Bartoli et al., 2015a, Potvin et al., 2015); or combination of

these effects. Our adiponectin secretion data for murine 3T3-F442A adipocyte model showed contradictory result (as discussed in chapter 2) compared to what showed clinically (Paredes et al., 2014, Pollmacher et al., 2000), which inclined us to use primary human adipocyte as a model to validate the effect of AAPs on lipid accumulation, adipogenic transcription markers, and adipocytokine release.

3.1.2 Hypothesis

This chapter tested the hypothesis that AAPs directly interfere with adipogenesis and adipocyte function which in turn result in AAP-induced weight gain and metabolic dysfunction.

3.1.3 Aims and objectives

The aims and objectives were;

1) To characterise AAP-induced metabolic toxicity *in vitro* using primary human adipocytes.

2) To investigate the mechanisms behind AAP-induced metabolic toxicity in primary human adipocytes by assessment of:

a. adipocyte lipid accumulation and gene transcription;

b. adipocyte-secreted protein markers and

c. adipocyte secreted inflammatory cytokines

3) To correlate the findings from Chapter 2 (3T3-F442A) with those generated using primary human adipocytes and thereby validate the murine model.

3.2 Methods

3.2.1 Materials

CLO and OLA were purchased from Sigma-Aldrich, MO, USA while ARI and LPV were obtained from Santa Cruz Biotechnology, TX, USA. Preadipocyte growth medium (supplemented with fetal calf serum [0.05ml/ml], endothelial cell growth supplement [0.004ml/ml], epidermal growth factor [10ng/ml], hydrocortisone [1µg/ml] and heparin [90µg/ml]), Differentiated growth medium (supplemented with insulin [0.5µg/ml], d-biotin [8µg/ml], d-dexamethasone [400ng/ml], 3-isobutyl-1-methylxanthine (IBMX) [44µg/ml], L-Thyroxine [9ng/ml] and ciglitazone [3µg/ml]), Nutritional growth medium (supplemented with fetal calf serum [0.03ml/ml], insulin [0.5µg/ml], d-biotin [8µg/ml] and d-dexamethasone [400ng/ml]), detach kit (containing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), trypsin and trypsin neutralising solution), and freezing medium were purchased from PromoCell, Heidelberg, Germany. DMSO, RIPA lysis buffer, chloroform and Oil Red O dye were purchased from Sigma-Aldrich, MO, USA. HBSS, phosphate-buffered saline (PBS), pre-cast gels for western blot, LDS sample buffer, sample reducing agent, enhanced chemiluminescent substrate, reverse transcription kit, Tri Reagent solution for RNA, FAM labelled PPARγ and lipin1, VIC labelled β- actin TaqMan gene expression assays, TaqMan gene expression master mix, isopropanol, human ELISA kits for adiponectin, IL-6, TNF-α, and leptin were purchased from Thermo Fisher Scientific, TX, USA. The nitrocellulose membrane was purchased from Gene Flow, Staffordshire, UK. All other cell culture materials were purchased from Thermo Fisher Scientific, TX, USA.

3.2.2 Cell Culture

Preadipocytes from abdominal subcutaneous adipose tissue obtained from healthy controls (n=3; Caucasian; age range: 31-43 years; Gender: female; BMI: 21-25kg/m^2) were obtained commercially from PromoCell, Heidelberg, Germany. The preadipocytes were cultured and maintained in a T75 flask in preadipocyte growth medium. Once confluent (75-80%), cell culture media was discarded and cells were washed carefully with 5ml of HEPES solution. 5ml of trypsin was added to the flask and incubated for 5 minutes at room temperature to dislodge the cells from the surface of the flask. After incubation cells were detached by gentle tapping of the bottom of the flask. 5ml of trypsin neutralization solution was added to neutralize trypsin. This mixture was then poured into a universal tube and centrifuged at 220g for 3 minutes at room temperature to obtain the cell pellet. The supernatant was discarded once centrifugation was finished and the cell pellet was suspended in 3-5ml of preadipocyte growth medium depending upon the size of the pellet. Cell counting was done by mixing 10µl of the cell containing media with 10µl of trypan blue dye. Then 10µl mixture of cells and dye was loaded into a Countess Cell chamber slide, and cell counting was performed using Cell Counter, Thermo Fisher Scientific, TX, USA. One million cells were then sub-cultured in a new T75 flask for maintenance of cell cohort. The remaining cells were either frozen or cultured in 6-well plates.

75,000 cells/well were seeded in 6-well plates containing preadipocyte growth medium. Once 70% confluent (usually take 3-5 days depending on the population doubling time for individual donor cells), differentiation was induced by replacing the basal medium with the differentiation medium (Counted as day 0 of differentiation). After 3 days, the differentiated media was replaced by adipocyte nutrition medium. The nutrition media was changed every 48hours for the next 10 days. Cells were treated with either vehicle or different doses of AAPs starting from day 3 to day 11

(total 5 drug additions). On day 13, 48 hrs after the last drug addition, conditioned media and cell lysates were collected to study adipocytokine levels, and gene and protein expression respectively. In our experiment, we used 3 donors to check the reproducibility of the results and for statistical purposes.

CLO (1μM, 20μM), OLA (0.2μM, 20μM) and ARI (0.2μM, 10μM) were used in the study. We focused primarily on two doses for each drug; one was a therapeutically used dose and the other was a higher dose (20uM) based on the assumption that AAPs might accumulate in the adipose tissue. LPV (20μM), an anti-HIV drug with known deleterious effects on adipocyte function was used as a comparator.

Cell death was minimal with a 20μM dose of CLO and OLA; however, 20μM ARI caused significant amount of cell death. The deleterious effect of 20μM ARI dose was also reported by Himmerich and colleagues (Himmerich et al., 2011) in their primary human adipocyte model. Because of this, we reduced the maximum concentration of ARI to 10μM.

Table 3.1. Drug treatment timeline

Preadipocytes **Preadipocyte media**			**Preadipocytes with DM media**	**Differentiated Adipocytes** **Adipocyte Nutrition Media**					
				Dose 1	**Dose 2**	**Dose 3**	**Dose 4**	**Dose 5**	**Collect lysates**
Day -3	**Day -2**	**Day -1**	**Day 0**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Day 13**

DM: Differentiating media

3.2.3 Lipid Accumulation

Lipid accumulation was performed by Oil Red O assay as described in section 2.2.4.

3.2.4 Measurement of Adipocytokine secretions

Adiponectin, IL-6, TNF-α and leptin secretion were measured according to manufacturer's protocol as described in section 2.2.5.

3.2.5 Gene and protein expression

Isolation of mRNA, reverse transcription, gene expression, and protein isolation and quantitation were measured by methodology as described in section 2.2.6 and 2.2.9

3.2.6 Western blotting

20µg protein was mixed with 5µl of a mixture of LDS sample buffer and sample reducing agent. This mixture was prepared by mixing 70µl of LDS sample buffer and 30µl of sample reducing agent. Samples were heated at 95°C for 5 min. All samples were centrifuged and kept on ice for further 5 min. Pre-cast gels were used for loading of the samples. Ladder and samples were loaded in their respective wells. The gel was run at 80 volts in running buffer until samples run past the stacking gel for 15 min and then 170 volts for 1-1.5 hr before the samples reach the ridge at the bottom of the cassette. The gel was then blotted in transfer buffer at 100V for 1hr during which proteins were transferred to nitrocellulose membrane. The membrane was blocked in 10% milk/TBST (Tris buffer saline-tween) solution for 1 hr to avoid non-specific binding of the antibodies. The primary antibody was diluted in 5% milk/TBST and the membrane was incubated with primary antibody overnight at 4 °C. After incubation, the nitrocellulose membrane was washed with TBST 6 times with repeated washes after every 5 minutes. After washing, the membrane was incubated with secondary antibody in 5% milk/TBST at room temperature for 1hr. The membrane was again washed 6 times with repeated washes after every 5 minutes. Enhanced chemiluminescent substrate was then applied and western blot was developed by using

ChemiDoc Touch Imaging system and quantitated by Image Lab software (Bio-Rad, CA, USA). The antibodies used were PPARγ (1:1000 dilution, catalogue number: 2443; Cell Signalling Technology, MA, USA), Anti-rabbit secondary antibody (1:2000 dilution: catalogue number 7074S: Cell Signalling Technology, MA, USA) and beta-actin (1:1000 dilution, catalogue number sc-47778: Santa Cruz Biotechnology, TX, USA).

3.2.7 Simple Western Assay

Simple Western was performed by methodology as described in section 2.2.11.

3.2.8 Clozapine Accumulation Assay

Both murine and human subcutaneous preadipocytes were cultured in 6 well plates. After reaching fully differentiated stage, (Cells kept in differentiating media, murine adipocytes: 10 days; primary human adipocytes: 3 days in differentiating media and then 10 days in adipocyte nutrition media), cells were washed two times with pre-warmed HBSS. Then 1ml/well pre-warmed master mix was added to each well. Master Mix was prepared which consist of transport buffer (HBSS supplemented with 25mM HEPES and 0.1% BSA; pH: 7.4) and 0.1µCi/ml tritium-labelled CLO with sufficient non-radiolabelled drug. A separate master mix was used for the non-specific transporter inhibitor, Verapamil which was used at a concentration of 100µM.The plates were incubated for 30min at 37°C. After incubation, cells were washed three times with ice-cold HBSS and then solubilised with 10% SDS for 30min.The lysates were finally transferred to scintillation tubes having 4ml scintillation cocktail and radioactivity was measured through Tri-Carb 1500 Time-Resolved Liquid Scintillation Analyzer, Packard, USA) (Dickens et al., 2013).

3.3 Statistical Analysis

All statistical comparisons (3 donors) were made using t-test on Stats Direct software version 2.7.9. Two-way repeated measure Analysis of variance (ANOVA) was conducted by Statistical Package for Social Sciences (SPSS) version 24. Differences were considered significant at $p \leq 0.05$.The result was shown as mean ± SD.

We have presented data relative to measured variables observed with the vehicle control. The following points have been deduced by going through all the data processed by two-way repeated measures ANOVA.

In all of our data, Mauchly's test of Sphericity indicated that the assumption of sphericity had not been violated for the one-way effects of treatment and dose or the interaction of the two (p=NS). Sphericity is the statistical condition where the variances of the differences between all combinations of related groups are equal. If it is not equal then there is a violation of sphericity. However, if data does not violate the assumption of sphericity then there is no need to modify degrees of freedom. Not violating this assumption means that the F-statistic that has been calculated is valid and can be used to determine statistical significance (https://statistics.laerd.com)(https://statistics.laerd.com/statistical-guides/sphericity-statistical-guide.php).

In all of our parameters, a test of between-subject effects showed that there is a statistically significant difference between the responses of the three donors in the study ($p \leq 0.05$). This indicates a high inter-individual variability. This showed that experiment itself worked fine with respect to drug effect on individual donor; however because of variability between donors (adipose tissue donors), we observed marked differences in the magnitude of the effect.

In two-way repeated measures ANOVA, we also performed comparison between therapeutic and high dose.

Overall, due to high inter-individual variability the results based on three samples should be utilised as indicators for further study as opposed to drawing a conclusion about the biology of the individual.

In conclusion, we can say that two-way repeated measures ANOVA analysis provide us detail information about our data and comparison which clearly indicated that the drug is producing some trend or change however due to inter-individual variability, most of the outcome became non-significant.

In result section, we will highlight our result along with two-way repeated measures ANOVA description separately for each parameter.

3.4 Results

3.4.1 Effect of AAPs on adipocyte lipid accumulation

We expressed lipid accumulation data for each drug and dose as a percentage of what was observed with the vehicle; this showed that CLO at therapeutic dose (1µM) resulted in significantly increased accumulation of lipids in differentiating adipocytes on day 13 (CLO; p=0.04) while therapeutic doses for OLA (0.2µM) and ARI (0.2µM) did not show any change. However, high doses of CLO (20µM) and OLA (20µM) but not ARI (10µM), showed a trend to increase lipid accumulation although it was not statistically significant (Fig.3.2A).

The two-way repeated measures ANOVA further confirmed the above results; statistically significant difference in the mean treatment response for lipid accumulation between treatments ($F(2, 4) = 7.07$, $p = 0.04$) was observed (The test statistic used for ANOVA is the F-statistic and is calculated by taking the Mean Square

(MS) for the variable divided by the MS of the error. This F-statistic is a ratio of the variability between groups compared to the variability within the groups). However, the difference in lipid accumulation between the high dose and therapeutic dose for all treatments were not statistically significant ($F(1, 2) = 0.431$, $p = 0.5$) (Fig.3.2B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4) = 1.882$, $p = 0.2$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p = 0.001$). However, individual donor response for lipid accumulation followed a similar trend for all the drugs tested (Fig.3.2C). Post hoc tests using the Bonferroni correction revealed that the therapeutic vs high dose effect elicited average 3% change in lipid accumulation, which was statistically non-significant (95% confidence Interval (CI) [18%, 25%], $p = 0.5$).

Oil Red O staining of primary human adipocytes also showed that CLO and OLA, at higher doses cause increased lipid droplets compared to ARI (Fig.3.3).

Fig 3.2A

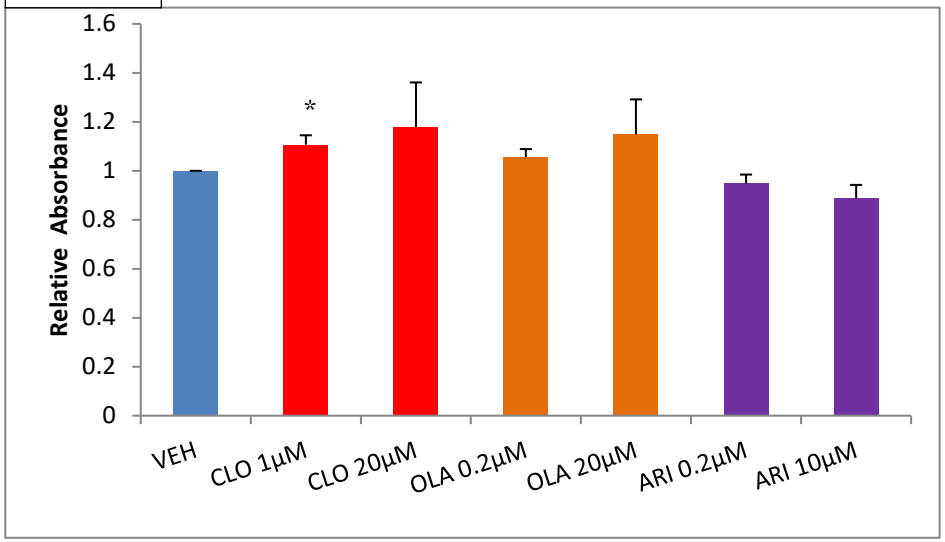


Fig 3.2B

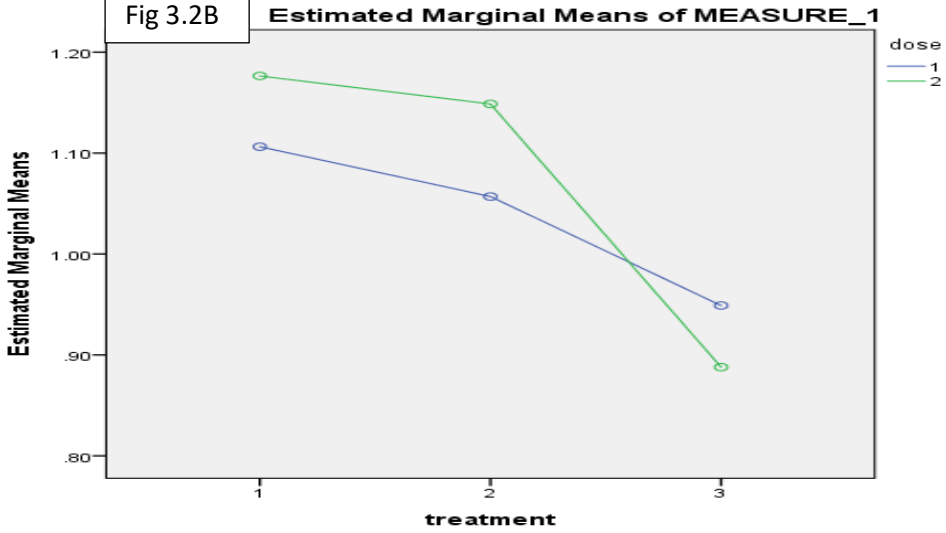


Fig 3.2C

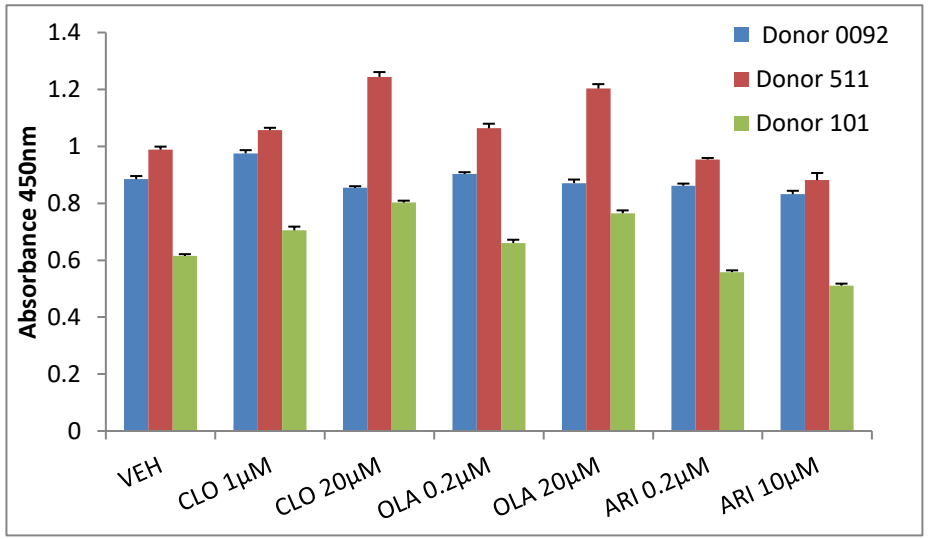


Figure 3.2: A) Quantitation of lipid-bound dye extracted by isopropanol showing the effect of various concentrations of CLO, OLA, and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05. * Vehicle vs CLO; B) Dose and Treatment Plot profile for lipid accumulation with AAPs. Dose 1(blue): therapeutic dose; Dose 2 (green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in lipid accumulation observed in individual donors with various concentrations of CLO, OLA and ARI.

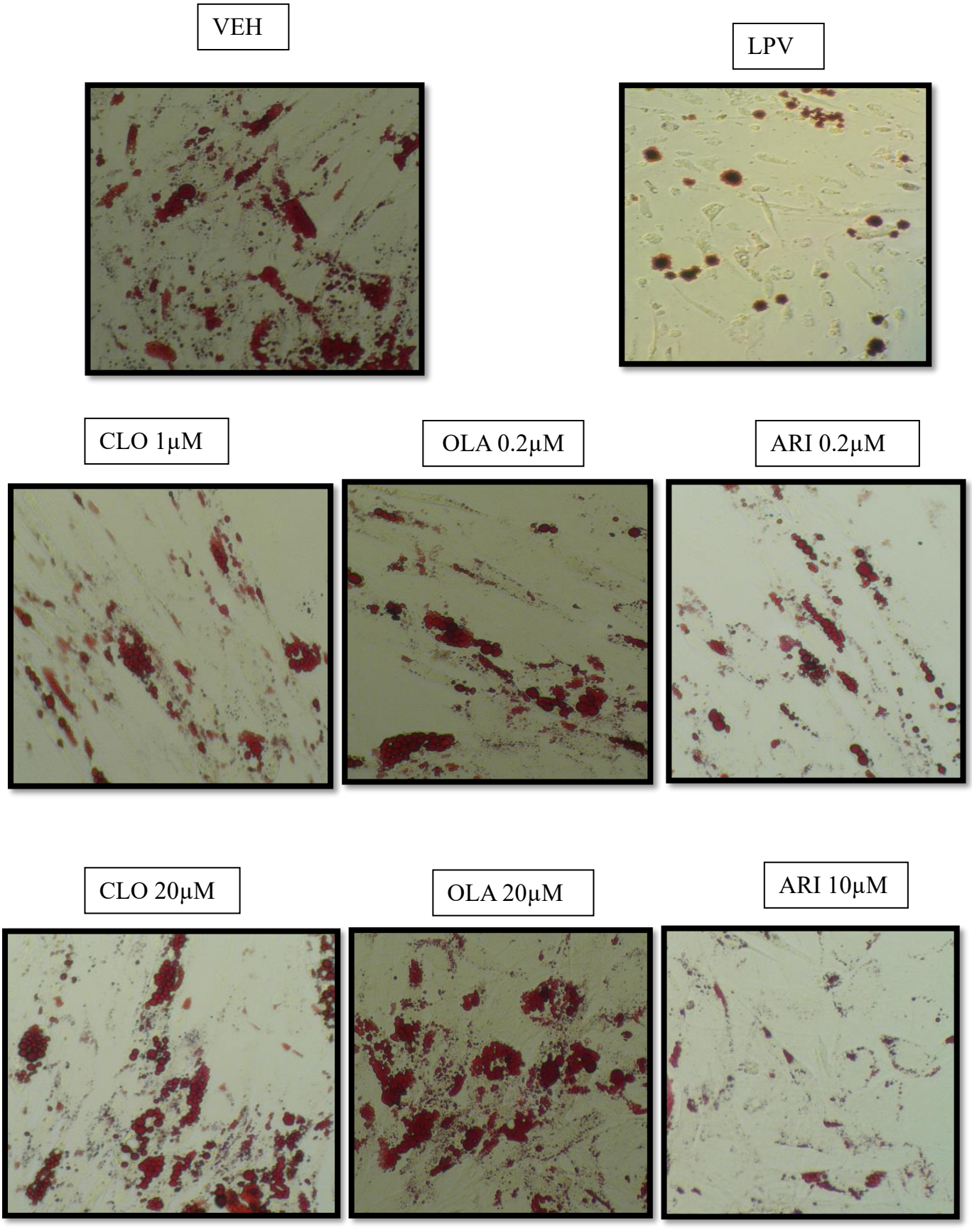


Figure 3.3. Photomicrographs (20x magnification) showing Oil Red O staining of cultured primary human adipocytes on 13th-day post differentiation treated with various concentrations of CLO, OLA and ARI.

3.4.2 Effect of AAPs on adipocytokines

3.4.2.1 Adiponectin

We expressed adiponectin levels for each drug and dose as a percentage of what was observed with the vehicle; this showed that both CLO and OLA at higher doses (20uM) resulted in significantly reduced secretion of adiponectin from the differentiating adipocytes on day 13 (CLO; p=0.03, OLA; p=0.01). However, there was no change in adiponectin secretion with the therapeutic doses of CLO or OLA. Both doses of ARI showed a trend to increase adiponectin although this was not statistically significant (Fig.3.4A).

The two-way repeated measures ANOVA further confirmed the above results; statistically significant difference in the mean treatment response for adiponectin secretion between treatments ($F(2, 4) = 10.16$, $p = 0.03$) was observed. There was also a statistically significant difference between high and therapeutic dose of all treatments ($F(1, 2) = 40.90$, $p = 0.02$) (Fig.3.4B). The analysis also showed that the interaction between treatment and dose was statistically significant ($F(2, 4) = 20.45$, $p=0.01$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment (p=0.003). However, individual donor response for adiponectin followed a similar trend for all the drugs tested (Fig.3.4C). Post hoc tests using the Bonferroni correction revealed that there was a 31% average change in adiponectin secretion between the therapeutic and high doses of individual AAPs which was statistically significant (95% CI, [10%, 52%], p =0.02).

Fig 3.4A

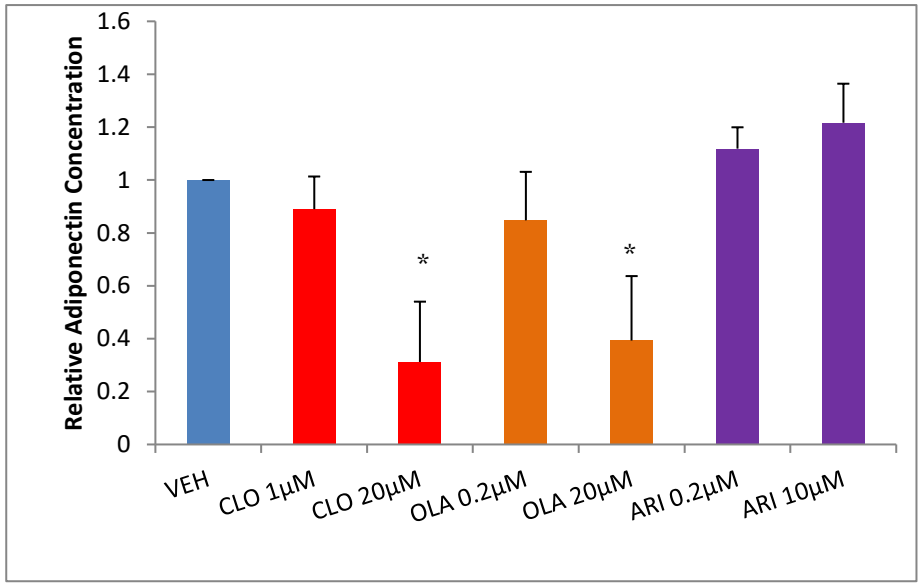


Fig 3.4B

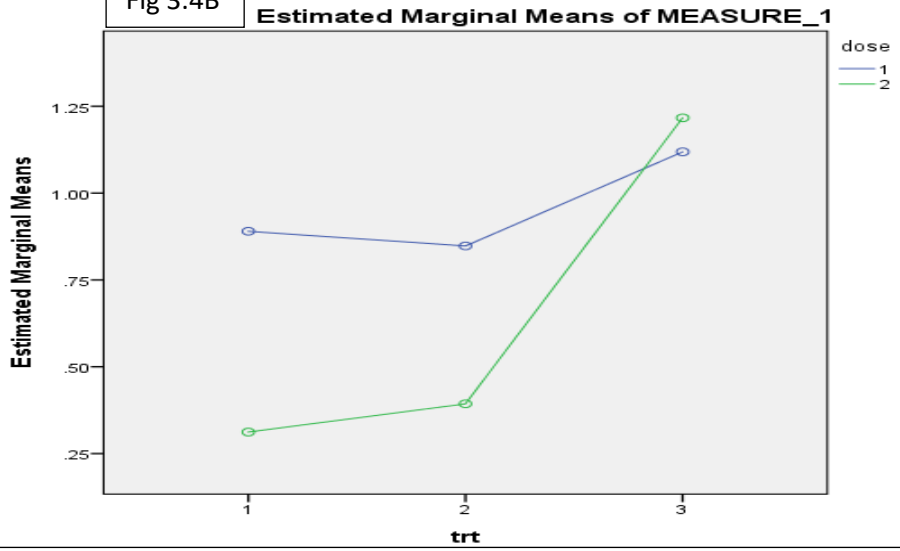


Fig 3.4C

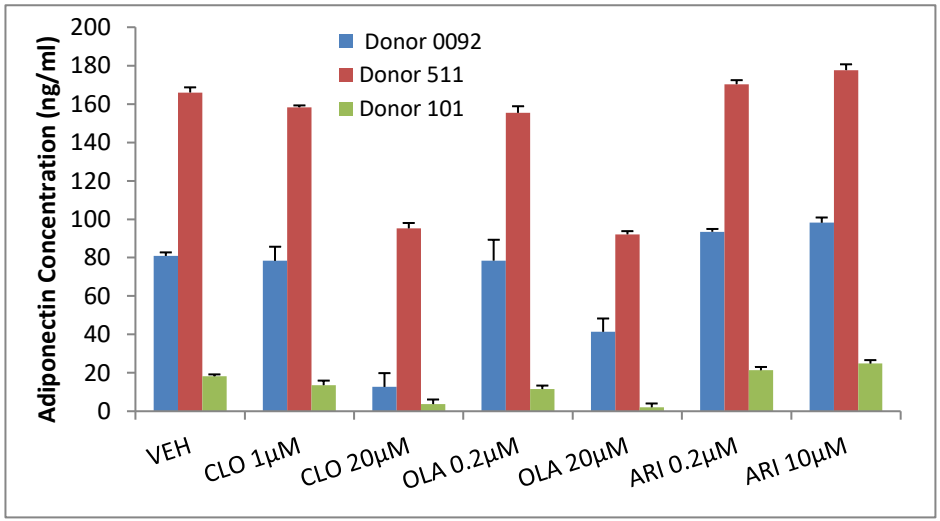


Figure 3.4: A) Adiponectin secretion showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ± SD; $p \leq 0.05$. * VEH vs CLO/OLA; B) Dose and Treatment Plot profile for adiponectin secretion with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in adiponectin secretion observed in individual donors with various concentrations of CLO, OLA and ARI.

3.4.2.2 Leptin

We expressed leptin levels for each drug and dose as a percentage of what was observed with the vehicle; this showed that CLO but not OLA resulted in significantly increased secretion of leptin from the differentiating adipocytes on day 13 (1µM CLO; p=0.04, 20µM CLO; p=0.01). Both doses of ARI showed a trend to decrease leptin secretions although this was not statistically significant (Fig.3.5A).

The two-way repeated measures ANOVA further confirmed the above results; statistically significant difference in the mean treatment response for leptin secretion between treatments ($F(2, 4) = 18.09$, $p=0.01$) was observed. However, the difference between high and therapeutic dose for all treatments were not statistically significant ($F(1, 2)=0.449$, $p=0.5$) (Fig.3.5B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4)=1.794$, $p=0.3$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p=0.001$). However, individual donor response for leptin followed a similar trend for all the drugs tested (Fig.3.5C). Post hoc tests using the Bonferroni correction revealed that there was only a 0.8% average change in leptin secretion between therapeutic and high dose used for various AAPs; this was statistically non-significant (95% CI, [4%, 5%], $p =0.5$)

Fig 3.5A

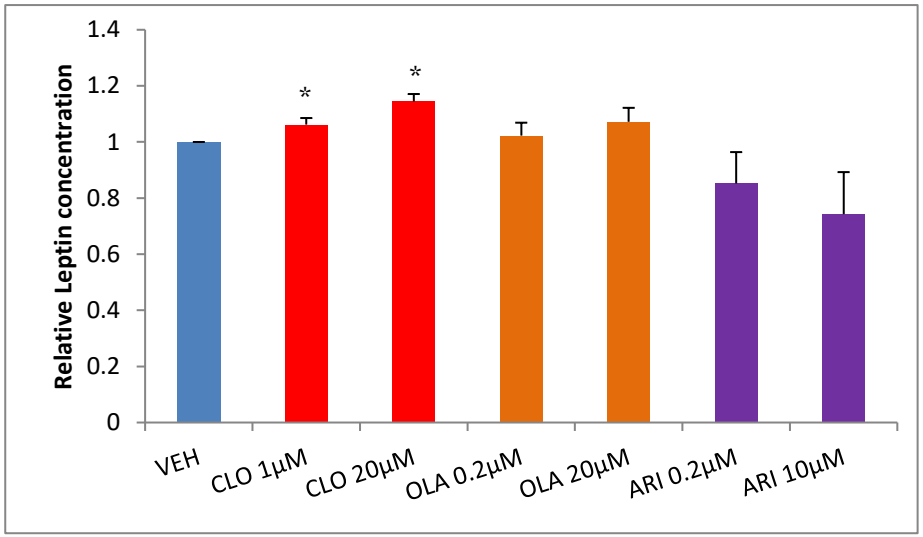


Fig 3.5B

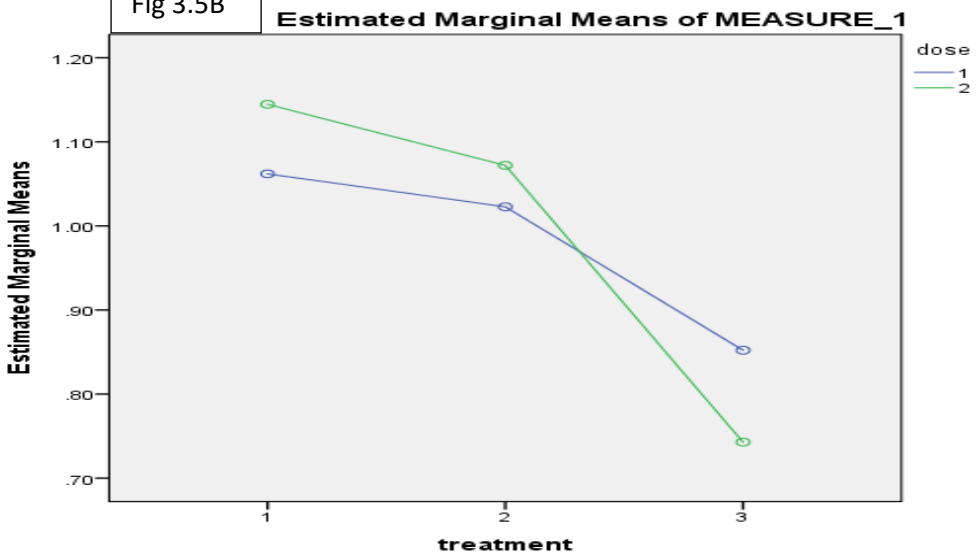


Fig 3.5C

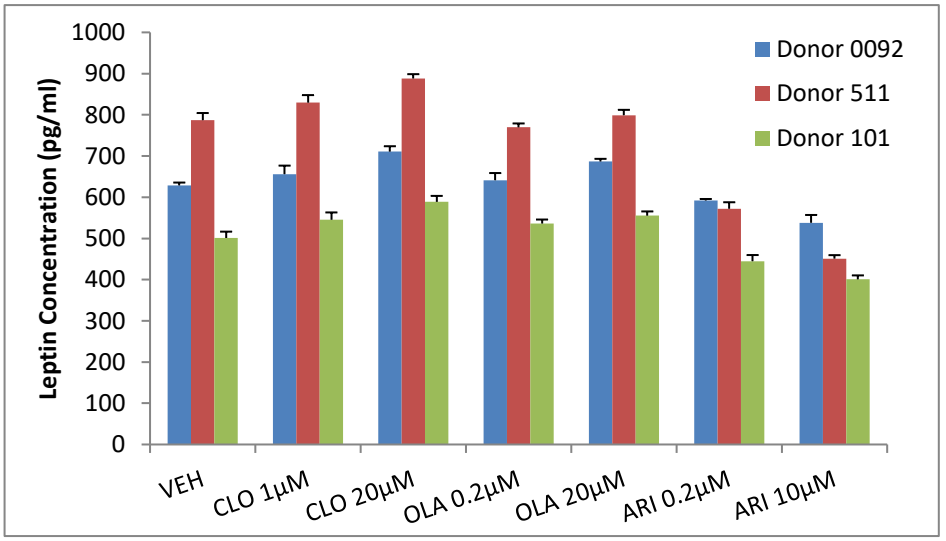


Figure 3.5: A) Leptin secretion showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05. * Vehicle vs CLO; B) Dose and Treatment Plot profile for leptin secretion with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in leptin secretion observed in individual donors with various concentrations of CLO, OLA and ARI.

3.4.2.3 IL-6

We expressed IL-6 levels for each drug and dose as a percentage of what was observed with the vehicle; this showed that the therapeutic doses of CLO, OLA and ARI did not show any change in IL-6 levels. However, high doses of both CLO and OLA but not ARI showed a trend to increase IL-6 secretion although this was not statistically significant (Fig.3.6A).

The two-way repeated measures ANOVA further confirmed the above results; no statistically significant difference in the mean treatment response for IL-6 secretion between treatments ($F(2, 4) = 1.67$, $p = 0.29$) was observed. The difference in IL-6 secretion between high and therapeutic dose for all treatments were also statistically non-significant ($F(1, 2) = 1.99$, $p=0.5$). (Fig.3.6B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4)=4.29$, $p=0.6$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p=0.005$). However, individual donor response for IL-6 followed a similar trend for all the drugs tested (Fig.3.6C). Post hoc tests using the Bonferroni correction revealed that there was a 6% average change in IL-6 secretion between the therapeutic and high dose tested for each AAP; but this was statistically non- significant (95% CI, [12%, 25%], $p =0.2$).

3.4.2.4 TNF-α

We did not detect TNF-α level in our samples despite using a high-sensitive TNF-α assay kit.

Fig 3.6A

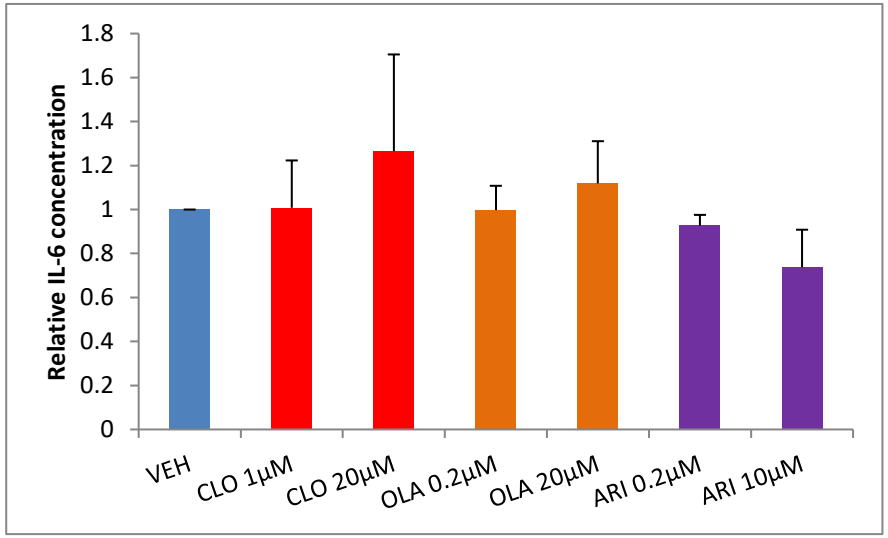


Fig 3.6B

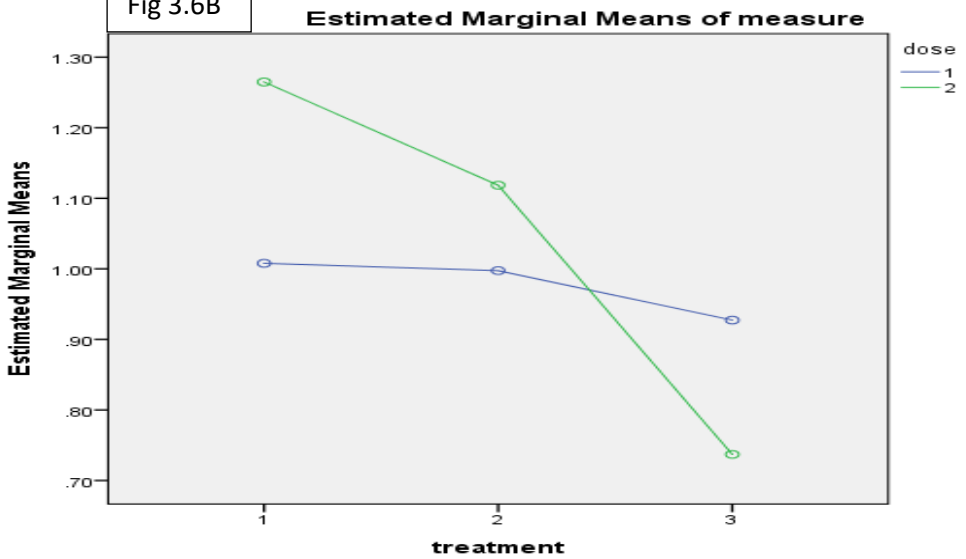


Fig 3.6C

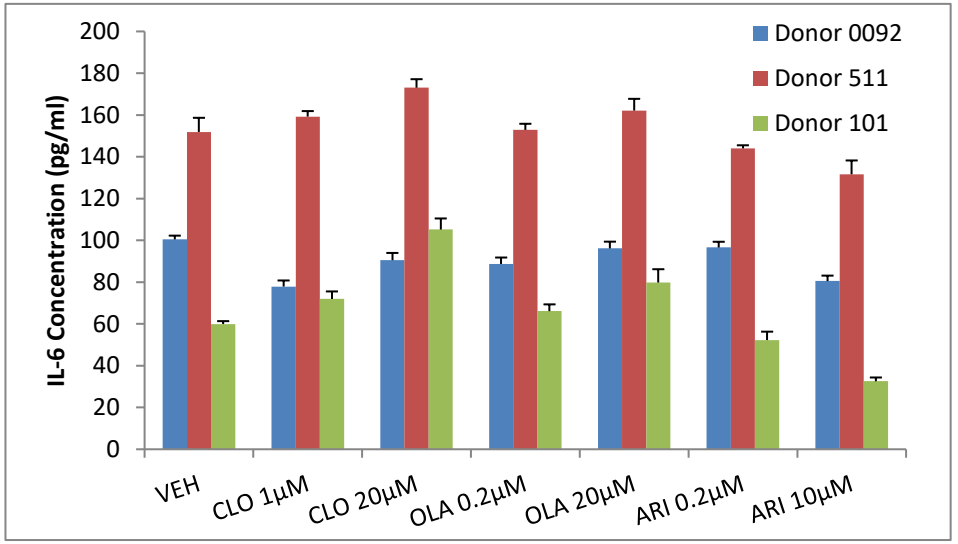


Figure 3.6: A) IL-6 secretion showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05; B) Dose and Treatment Plot profile for IL-6 secretion with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in IL-6 secretion observed in individual donors with various concentrations of CLO, OLA and ARI.

3.4.3 Effect of AAPs on PPARγ and lipin1 gene and protein expression

3.4.3.1 *PPARγ* gene

We expressed *PPARγ* mRNA expression for each drug and dose as a percentage of what was observed with the vehicle; this showed that neither CLO nor OLA showed any change in expression. ARI, on the other hand, showed a trend to increase *PPAR*γ expression at therapeutic dose but this was statistically non-significant. There was no change in *PPAR*γ gene expression at 10μM of ARI (Fig.3.7A).

The two-way repeated measures ANOVA further confirmed the above results; no statistically significant difference in the mean treatment response for *PPARγ* gene expression between treatments ($F(2, 4) = 0.286$, $p = 0.7$) was observed. No difference in *PPAR*γ expression was observed between high and therapeutic dose for all treatments ($F(1, 2) = 0.50$, $p = 0.5$) (Fig.3.7B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4) = 0.382$, $p = 0.7$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p = 0.04$). However, individual donor response for expression followed a similar trend for all the drugs tested (Fig.3.7C). Post hoc tests using the Bonferroni correction revealed that there was a 15% average change in *PPAR*γ gene expression between the therapeutic and high dose tested for AAPs; however, this was statistically non- significant (95% CI, [79%, 110%], $p = 0.5$).

3.4.3.2 PPARγ protein expression

We expressed PPARγ protein expression for each drug and dose as a percentage of what was observed with the vehicle; this showed that although both doses of CLO and

the high dose of OLA showed a trend to increase PPARγ protein expression, this was not statistically significant. Neither dose of ARI showed any difference in PPARγ expression (Fig.3.8A).

The two-way repeated measures ANOVA further confirmed the above results; no statistically significant difference in the mean treatment response for PPARγ protein expression between treatments ($F(2, 4) = 0.456$, $p = 0.6$) was observed. Also, there was no difference between high and therapeutic dose for all treatments ($F(1, 2) = 0.313$, $p=0.6$) (Fig.3.8B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4)=0.155$, $p=0.6$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p=0.03$). However, individual donor response for expression followed a similar trend for all the drugs tested (Fig.3.8C). Post hoc tests using the Bonferroni correction revealed that the therapeutic vs high dose effect elicited on average 7% change in expression, which was statistically non- significant (95% CI, [51%, 67%], $p =0.6$).

Fig. 3.7A

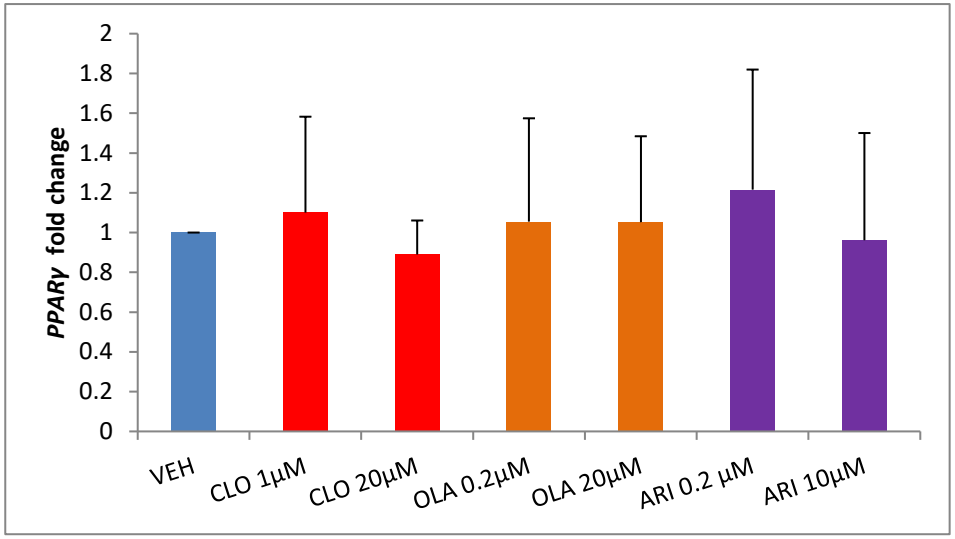


Fig. 3.7B

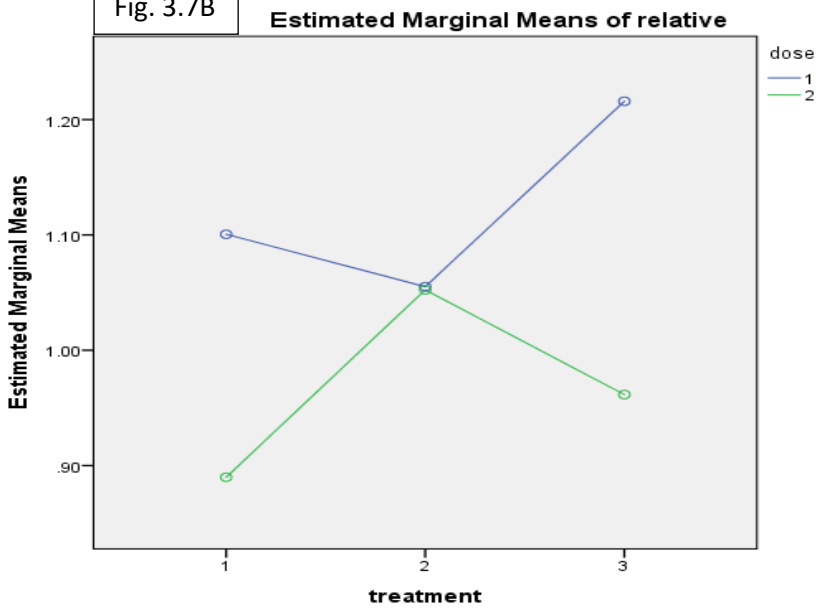


Fig. 3.7C

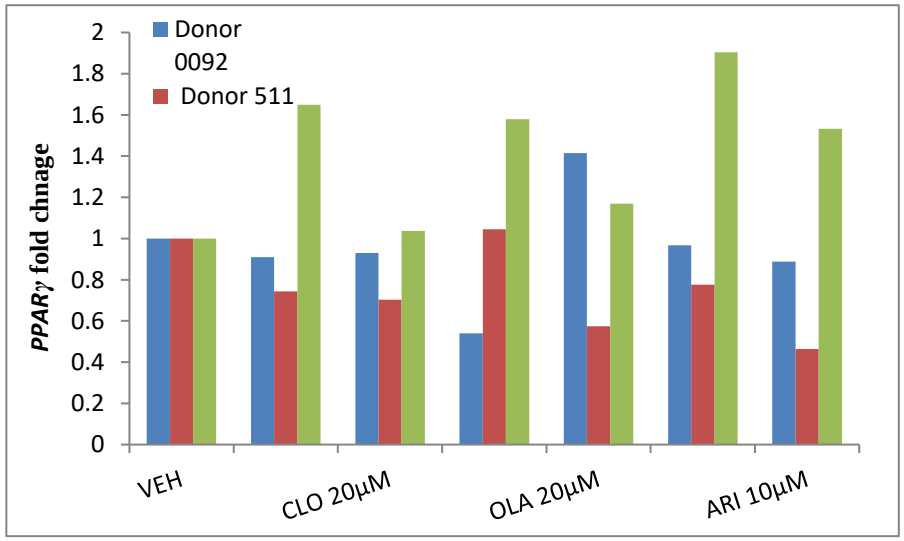


Figure 3.7: A) *PPARγ* gene expression showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05; B) Dose and Treatment Plot profile for *PPARγ* gene expression with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in *PPARγ* gene expression observed in individual donors with various concentrations of CLO, OLA and ARI.

Fig. 3.8A

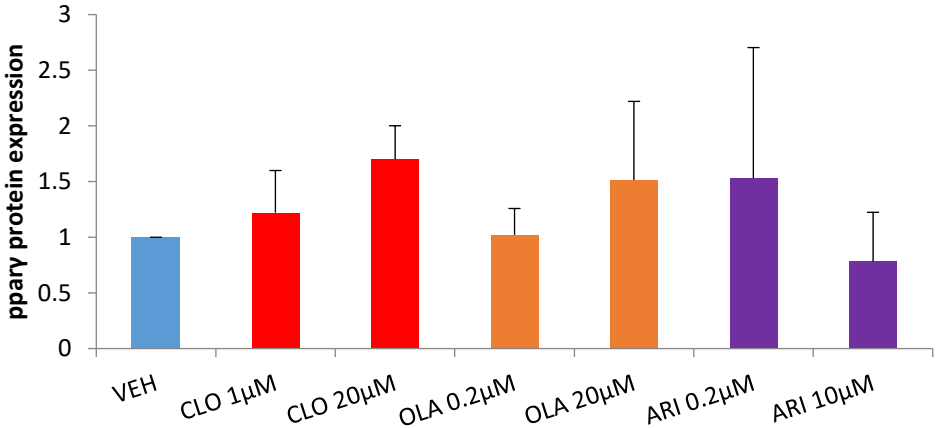


Fig. 3.8B

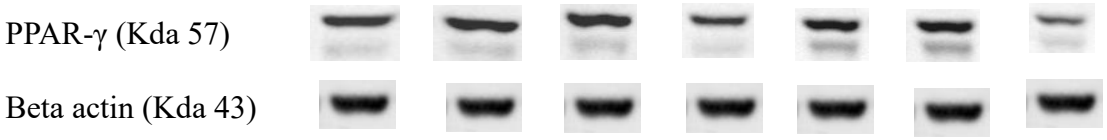
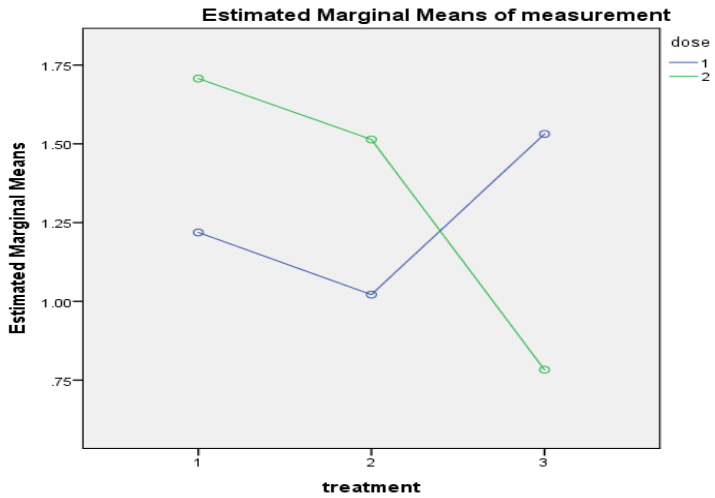


Fig. 3.8C



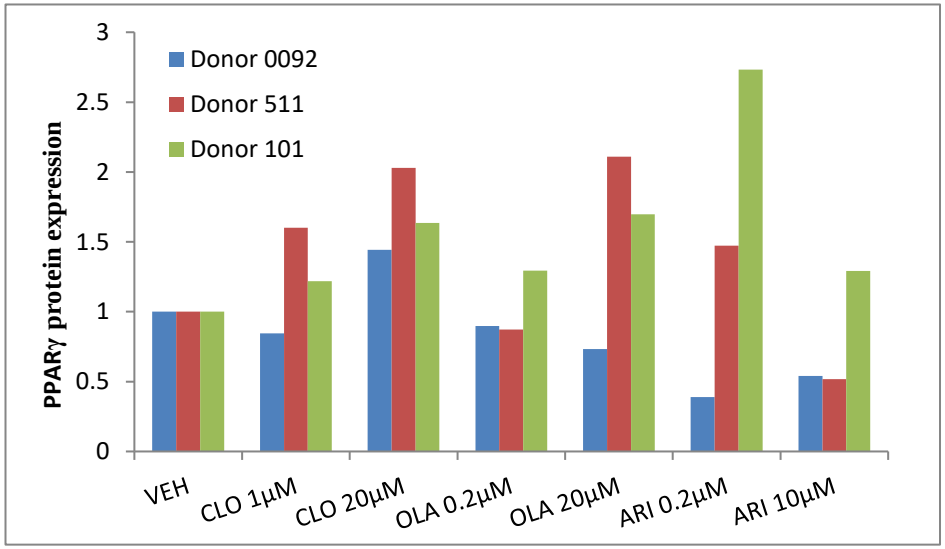


Figure 3.8: A) PPARγ protein expression showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05; B) Dose and Treatment Plot profile for PPARγ protein expression with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in PPARγ protein expression observed in individual donors with various concentrations of CLO, OLA and ARI.

3.4.3.3 *LPIN1* gene expression

We expressed *LPIN1* mRNA expression for each drug and dose as a percentage of what was observed with the vehicle; this showed that therapeutic doses of all three AAPs showed no change in expression. Interestingly, higher dose of all AAPs showed a trend to decrease *LPIN1* mRNA expression however this was not statistically significant (Fig.3.9A).

The two-way repeated measures ANOVA further confirmed the above results; no statistically significant difference in the mean treatment response for *LPIN1* gene expression between treatments ($F(2, 4) = 0.068$, $p = 0.9$) was observed. No difference was observed between high and therapeutic dose for all treatments ($F(1, 2) = 0.55$, $p=0.4$) (Fig.3.9B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4)=1.049$, $p=0.4$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p=0.001$). However, individual donor response for expression followed a similar trend for all the drugs tested (Fig.3.9C). Post hoc tests using the Bonferroni correction revealed that the therapeutic vs high dose effect elicited on average 31% change in expression, which was statistically significant (95% CI, [13%, 49%], $p =0.01$).

3.4.3.4 LPIN1 protein expression

We expressed LPIN1 protein expression for each drug and dose as a percentage of what was observed with the vehicle. Although there were some trends to increase/decrease LPIN1 protein expression between the doses tested, none of the changes observed were statistically significant (Fig.3.10A).

The two-way repeated measures ANOVA further confirmed the above results; no statistically significant difference in the mean treatment response for LPIN1 protein expression between treatments ($F(2, 4) = 5.95$, $p = 0.06$) was observed; No difference was observed between high and therapeutic dose for all treatments ($F(1, 2) = 1.38$, $p=0.3$) (Fig.3.10B). The analysis also showed that the interaction between treatment and dose was statistically non-significant ($F(2, 4)=0.954$, $p=0.4$).

Two-way repeated measures ANOVA also showed that there was significant difference between subjects studied in this experiment ($p=0.02$). However, individual donor response for expression followed a similar trend for all the drugs tested (Fig.3.10C). Post hoc tests using the Bonferroni correction revealed that the therapeutic vs high dose effect elicited on average 27% change in expression, which was statistically non- significant (95% CI, [72%, 126%], $p =0.3$).

Fig 3.9A

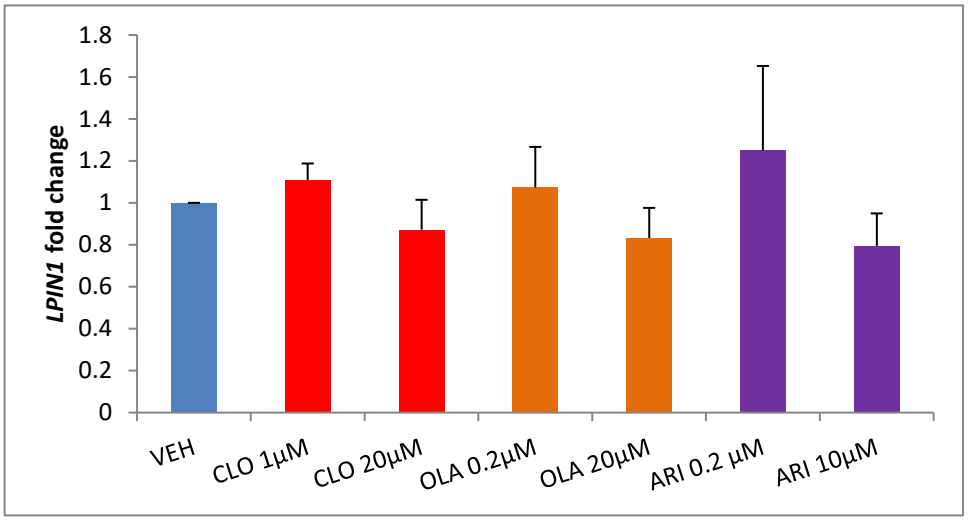


Fig 3.9B

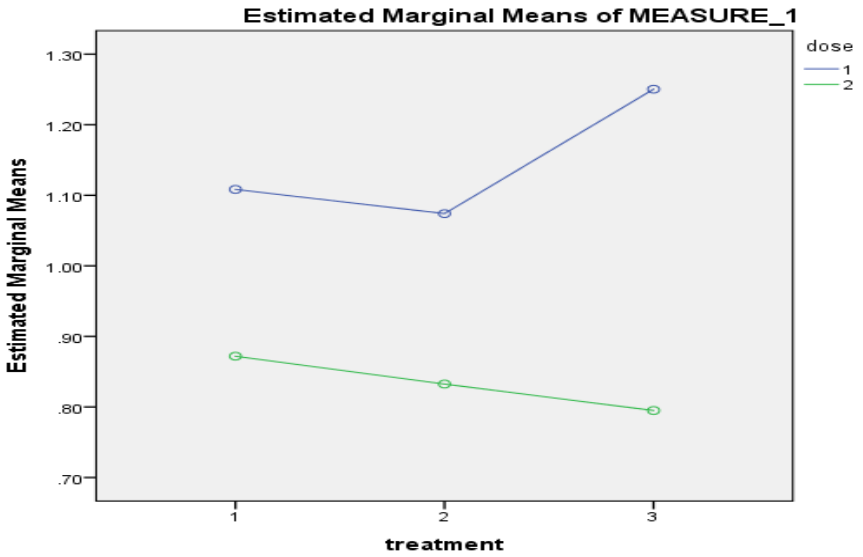


Fig 3.9C

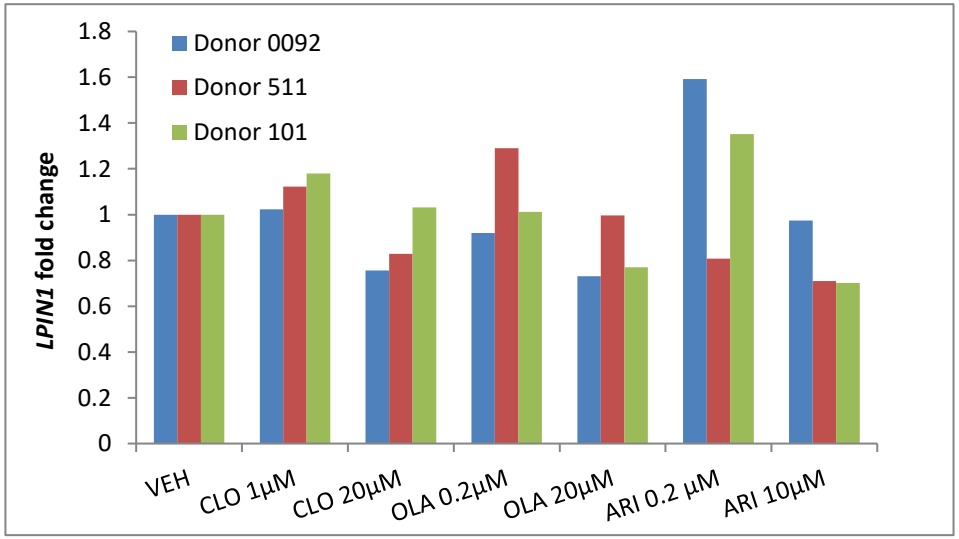
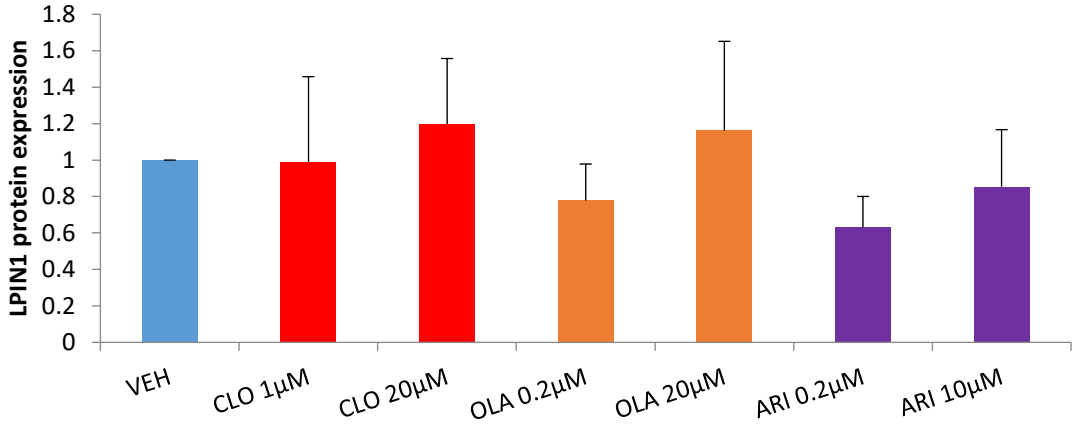


Figure 3.9: A) *LPIN1* gene expression showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05; B) Dose and Treatment Plot profile for *LPIN1* gene expression with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in *LPIN1* gene expression observed in individual donors with various concentrations of CLO, OLA and ARI.

Fig. 3.10A



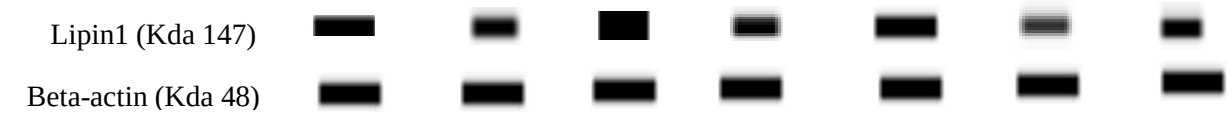


Fig. 3.10B

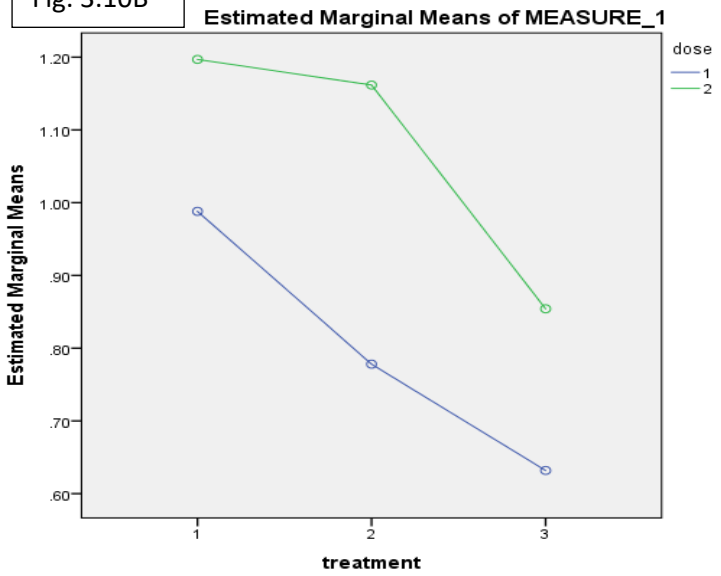


Fig. 3.10C

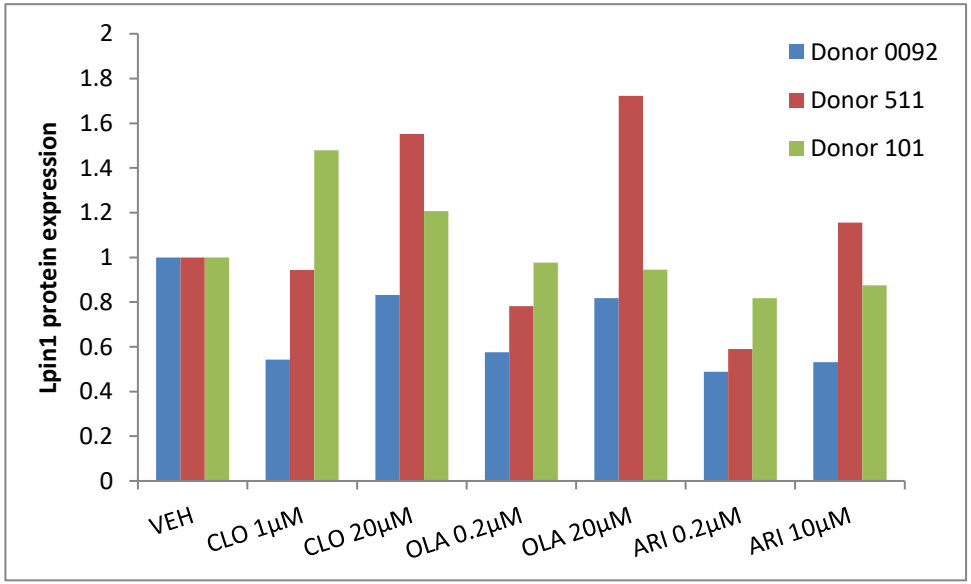


Figure 3.10: A) LPIN1 protein expression showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as Mean ±SD; p≤0.05; B) Dose and Treatment Plot profile for LPIN1 protein expression with AAPs. Dose 1(blue): therapeutic dose; Dose 2(green): higher dose; Treatment 1: CLO, 2: OLA, 3: ARI; C) Change in LPIN1 protein expression observed in individual donors with various concentrations of CLO, OLA and ARI.

3.4.4 Clozapine accumulation Assay

We observed an increase in adiponectin secretion by CLO in our experiment on 3T3-F442A murine adipocytes (discussed in Chapter 2). This contradicted with already published *in vitro* data (Tsubai et al., 2017) as well as clinical data (Sugai et al., 2012, Klemettila et al., 2014) which reported decrease adiponectin levels by CLO. To investigate this discrepancy, we used primary human adipocyte as another model to study the effect of CLO on adiponectin. We compared the accumulation of CLO in murine and primary human adipocytes to investigate whether there was any change in the uptake of the drug between these two cell models.

Our results showed that CLO accumulated 20-fold more in primary human adipocytes compared to the 3T3-F442A murine adipocytes (Fig.3.11 A). On adding verapamil, a nonspecific multitransporter inhibitor, there was a significant decrease in the accumulation of CLO (593±158.01; p=0.006) in primary subcutaneous human adipocytes compared to the control (1293.4±170.56) (Fig. 3.11B) whereas in 3T3-F442A adipocytes there was no change (45.63±26.96; p=ns) compared to control (64.26±15.17) (Fig.3.11 C).

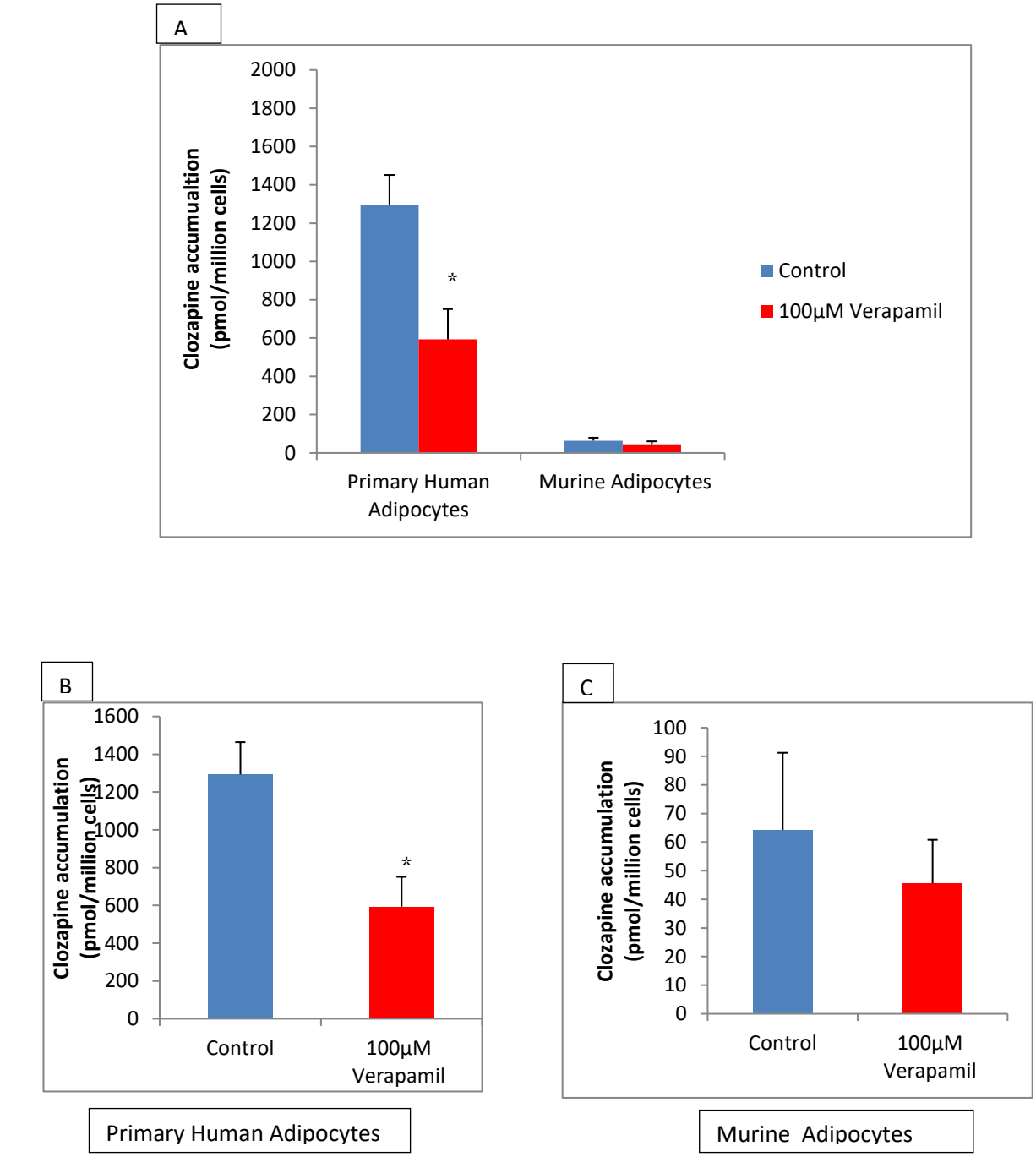


Fig 3.11: A) Accumulation of CLO in 3T3-F442A differentiated adipocytes and subcutaneous human adipocytes. Cells were incubated for 30min in the master mix with 1µM of CLO (H3) in the presence or absence of transport inhibitor (100µM Verapamil); B) Accumulation of CLO in adipocytes cultured from 3 individual donors; C) Accumulation of CLO in 3T3-F442A differentiated adipocytes which was repeated 3 times. Data was shown as Mean± SD; p≤0.05. *Control vs Verapamil

3.5 Discussion

Various mechanisms have been highlighted to be involved in AAP-induced metabolic adverse effects however; there is a need to address the exact mechanisms by which this toxicity happens. This is important as it will guide therapeutic decisions and also lead to the development of new drugs with minimal adverse effects. Various studies have proposed a direct effect of AAPs on the adipose tissue leading to an increase in lipid accumulation. This increase is considered as one of the mechanisms causing metabolic toxicity (Minet-Ringuet et al., 2007, Pavan et al., 2010, Hemmrich et al., 2006). Our data showed a significant increase in lipid accumulation by CLO at a therapeutic dose (1µM) while higher doses of both CLO and OLA, even though a trend to increase was observed, the result was non-significant. On the other hand, ARI showed a trend to decrease or no change. Our findings with CLO and OLA are consistent with the previous studies which showed an increase in lipid accumulation with CLO in human adipocytes isolated from subcutaneous adipose tissue (Hemmrich et al., 2006, Pavan et al., 2010) and non-significant increase in lipid accumulation observed with OLA in adipose-derived stem cells isolated from abdominal subcutaneous adipose tissue (Sertie et al., 2011). Hemmrich and colleagues reported a decrease in lipid accumulation with ARI in primary human adipocytes (Hemmrich et al., 2011); our own results are in agreement with these results. Increase in lipid accumulation observed in our study with CLO particularly may suggest that this could be one of the mechanisms by which CLO (and to certain extent, OLA) result in increased weight gain. However it must be highlighted that there are other studies which has failed to show any effect by CLO on adipogenesis (Hauner et al., 2003). Hauner's study used mammary-derived adipose tissue rather than subcutaneous adipose tissue; regiospecific differences in the way adipose tissue respond to drugs has

been shown before and could be one of the reason for differences observed between this study and Hauner's study. Also, Hauner et al exposed the cells to CLO only for 24hrs; this study utilised a chronic dosing pattern with long term drug incubations (drug exposure once every 48 hrs for a period of 10 days) to mimic the clinical model. This could also be the reason for the discrepancy in results.

Adipogenesis is driven by several transcription factors and their downstream target genes which are important for glucose and lipid metabolism. Some of these factors are also important for maturation and maintenance of adipocytes. Among these, the most important are PPARγ and lipin1. Even though we observed a trend to increase for PPARγ and lipin1 proteins, we encountered high in-between variability in response between our biological replicates and therefore these results were not statistically significant. We only used 3 biological replicates because of i) limitations in the availability of adipocytes from donors who met our inclusion criteria, and, ii) logistics of performing a chronic toxicity experiment in a larger number of replicates. It may be that an increased sample size could have resulted in reduced variability. However, when individual samples are analysed in isolation, the trend observed in expression of PPARγ and lipin1 protein (increased expression with higher dose of CLO and OLA while ARI reduced it) was consistent between all 3 biological replicates. In addition, our results on *PPAR*γ gene tallies with previous studies which showed no change in *PPAR*γ with therapeutic dose of CLO. Our data also coincides with previous studies where OLA (Nimura et al., 2015, Sertie et al., 2011) and ARI (Sarvari et al., 2014) showed no change in PPARγ mRNA expression. This suggests a role for other mechanisms involved in AAP-induced metabolic adverse effects. We have for the first time investigated the expression of cellular lipin1 and whether it change with AAPs. However, unlike PPARγ, the results varied between the biological replicates for

individual doses of AAPs and it was difficult conclude how lipin1 expression changes with AAPs. Future studies with an increased sample size are required to explore this transcription factor as it is important for maturation and maintenance of adipocytes and functions as a co-factor between PPARγ and CEBP-α to produce target genes necessary for the maintenance of adipocytes (Reue and Zhang, 2008). Adiponectin is an insulin-sensitizing cytokine, the lower levels of which caused insulin resistance. Our data showed a decrease in adiponectin secretion by CLO and OLA but not ARI. This coincides with clinical studies which have reported reduction in adiponectin levels with CLO and OLA but not with ARI (Paredes et al., 2014, Pollmacher et al., 2000, Klemettila et al., 2014, Hanssens et al., 2008). Human studies showed negative correlation between adiponectin and insulin sensitivity. It has been observed that adiponectin levels were decreased in subjects having insulin resistance in condition like T2DM or obesity (Weyer et al., 2001, Jin et al., 2008). It has also been showed that treatments given to improve insulin sensitivity also improved circulating adiponectin levels (Arita et al., 1999, Wang et al., 2006). This suggests that CLO and OLA may act directly on adipocytes and decrease the secretion of adiponectin which ultimately cause insulin resistance. Leptin is another important adipokine which has been suggested to play a role in metabolic disease. Leptin has a role in energy balance and suppresses food intake and thereby cause weight loss (Lu et al., 2015). Our leptin results showed a significant increase in secretion by CLO but not OLA. However, ARI showed a trend to decrease. Our data coincides with clinical data in terms of CLO and ARI (Paredes et al., 2014, Jin et al., 2008); however, it did not show a significant increase in leptin levels with OLA as suggested by previous studies (Potvin et al., 2015). This could be because of a variety of reasons. Firstly, our data comes from only 3 donors and therefore does not fully represent the wider population. Secondly, it has

been proposed that some individuals might be resistant to drug effect on leptin or vary in sensitivity for leptin metabolism. Increase in concentration of leptin without food reduction in obese patients had been reported suggesting the development of leptin resistance in these patients (Potvin et al., 2015). This showed that AAPs could exacerbate leptin resistance condition. This resistance could generate metabolic condition which leads to weight gain.

Another important mechanism suggested for the development of drug-induced metabolic toxicity is inflammation within the adipose tissue. It has been suggested that proinflammatory cytokines like IL-6 or TNF-α upregulate the suppressor of cytokine signalling 3 (SOCS3) which act on IRS1 and block its phosphorylation leading to insulin resistance (Makki et al., 2013). IL-6 levels showed a trend to increase with both CLO and OLA (particularly for the higher doses) but not ARI although these were statistically non-significant. Our results are consistent with what is observed with clinical studies (Leonard et al., 2012, Sobis et al., 2015). This might suggest inflammation as one of the main potential mechanisms by which CLO and OLA cause insulin resistance leading to metabolic toxicity. We did not detect any TNF-α in our conditioned media despite using a high-sensitive TNF-α assay kit. This might suggest that only negligible amounts of TNF-α was present in the conditioned media. However this contradict with clinical results which showed increased levels of TNF-α in AAP-treated schizophrenic patients (Klemettila et al., 2014, Pollmacher et al., 2000). However *in vitro* study on isolated subcutaneous human adipocytes by Sarvari et al showed, despite detection of TNF-α level, no significant change in levels by CLO, OLA and ARI. One possibility of contradictory data in our study might be less exposure of cells to drugs compare to Sarvari et al methodology where they add drugs continuously for 11 days and changed conditioned media daily while we exposed cells

to drugs for only 5 times with change of conditioned media after every 48hr. Clinically AAPs were also used for long period of time due to which TNF-α levels were easily detected.

Overall, our model showed results which broadly coincided with clinical studies performed previously as well as results from other *in vitro* data. Whilst we saw a trend to change for various markers tested, we were unable to show a concrete change in some of the markers largely due to variability observed with our 3 biological replicates. Our results clearly show that AAPs produce a direct effect on adipocytes and interfere with adipocyte lipid accumulation and secretion of adiponectin and leptin. Higher doses of CLO and OLA also result in changes in PPARγ and lipin1 protein expression. Importantly our results show that there is a distinct difference between the metabolically high risk AAPs (CLO and OLA) and ARI, an AAP which is not associated with metabolic toxicity.

Our data also showed high variability between adipocytes obtained from individual donors. However, if we consider each donor separately, it can be observed clearly that the patterns of gene and protein changes elicited by AAPs are almost similar. The variability between individuals may be due to various factors such as age, gender, hormonal changes and difference in receptor distribution between adipocytes obtained. Variability could also be there in the amount of drug metabolising enzymes between individuals. The variability was also seen in preclinical experiments where most studies (Choi et al., 2007, Davoodi et al., 2009, Boyda et al., 2010, Kalinichev et al., 2005, Lykkegaard et al., 2008, Ota et al., 2002, Park et al., 2008, Patel et al., 2009, Raskind et al., 2007, Sondhi et al., 2006) reported significant increase in weight gain, while few showed no effect (Amamoto et al., 2006, Baptista et al., 2002, Fell et al., 2008, Fell et al., 2005, Lacruz et al., 2000, Minet-Ringuet et al., 2007) or minimal

change (Cooper et al., 2007, Cooper et al., 2008, Lin et al., 2006, Pouzet et al., 2003). A gender specific difference was observed in rats when tested with OLA; female rats showed weight gain but this was not observed with male rats. On the other hand, CLO generally showed a negative result in both genders in rats showing gender-dependent effects as well (Panariello et al., 2011). Covell et al study on schizophrenic patients reported that AAPs especially CLO and OLA cause more weight gain in female patients than male showing gender specific differences (Covell et al., 2004).

Our findings also coincided with other researchers who used less number of donors and reported higher variation in their result which clearly depicts that variation could be due to donor itself and not due to experimental errors (Sarvari et al., 2014, Sertie et al., 2011).

We obtained contradictory results for adiponectin secretion for the murine and primary human adipocyte models. This prompted us to measure the uptake of CLO as an exemplar AAPs into both these cell types. Our CLO accumulation assay data showed 20-fold more accumulation of CLO in primary human adipocytes than the murine adipocytes. This would mean that the murine adipocytes were only getting exposed to a very low concentration of the drug despite addition of the same concentration of CLO to both murine and primary human adipocyte cultures. The contradictory result we found for adiponectin could be potentially explained on the basis of this. This might suggest differences in the uptake of AAPs by different type of cells (murine and human adipocytes); this may include differences in both transporter-dependent and non-dependent mechanisms which should be carefully considered before selecting an *in vitro* model.

In summary, our data demonstrated high inter-individual variability between the 3 adipocyte donors which limits statistical significance of the results, and the

conclusions that can be drawn. In future studies a large number of donors may decrease the variability. Our data also suggest that the primary human adipocyte is a better translational model than the murine 3T3-F442A preadipocyte model.

The primary human adipocytes data coincided with the clinical data observed for adiponectin; this made us to choose primary human adipocytes as the preferred model in subsequent experiments which will be discussed in chapter 4 and chapter 5.

Chapter 4

Therapeutic strategies to reverse AAP-induced metabolic adverse effects in *in vitro* primary human adipocyte model

4.1 Introduction

AAPs cause various metabolic adverse effects, including weight gain and insulin resistance, which are often difficult to treat. However various therapeutic strategies have been suggested as an option to reverse these metabolic adverse effects. There is little data available regarding the use of adjunctive drugs to reverse AAP-induced metabolic adverse effects (Mizuno et al., 2014). Schizophrenia treatment guidelines recommend antipsychotic monotherapy, but suggest polypharmacy as the last option to control metabolic adverse effects. Many combinations have been used to reduce psychotic symptoms plus metabolic adverse effects (Fleischhacker and Uchida, 2014). The most common combination used has been CLO and OLA with other AAPs or with other drug groups which include nizatidine, amantadine, reboxetine, topiramate (TOPI), sibutramine, metformin (MET), rosiglitazone (ROSI), ARI, phenylpropanolamine, modafinil, fenfluramine and atomoxetine. Data from various randomised clinical trials showed better results or improvements in metabolic adverse effects when AAPs were used together with MET, ARI, TOPI and ROSI (Zimbron et al., 2016). A review conducted by Faulkner et al showed improvement in metabolic adverse effects after using reboxetine and TOPI as an adjunctive, but showed mixed results for amantadine, nizatidine and sibutramine (Faulkner et al., 2007). They also proposed that adjunctive therapies should be applied to antipsychotic patients who followed non-pharmacological approaches, like physical activity and diet restriction for managing AAP-induced metabolic adverse effects. Their studies also highlighted some limitations due to small sample size, short duration of the study, the variability of the interventions, failure to study the adverse effects of add-on drugs, pharmacokinetic effects and failure to study specific mechanism by which drugs reduced metabolic adverse effects (Faulkner et al., 2007). Another review by Maayan and colleagues showed significant improvement in metabolic adverse effects,

especially body weight, by using concomitant medications which included sibutramine, TOPI, reboxetine, MET and D-fenfluramine. They found that, among all the drugs used, MET showed the strongest effect on weight and other metabolic parameters. However, their study could not find sufficient evidence to make recommendations for broad clinical use due to the small sample size, short study duration and the non-availability of data on the adverse effects caused by adjunctive drugs (Maayan et al., 2010). The research by Mizuno and colleagues also favoured MET as more effective in reducing weight in CLO and OLA-treated schizophrenic patients compared to TOPI, ARI, sibutramine, and reboxetine. It was also reported that MET and ROSI improved insulin resistance, while ARI, MET and sibutramine decreased cholesterol and triglyceride levels more effectively than all the other drugs used (Mizuno et al., 2014). From this report, we can surmise that MET seems to be the first choice to use as an add-on medication in AAP-treated schizophrenic patients as it showed improved effects on body weight, lipids and insulin resistance. However, a meta-analysis by Choi suggested that TOPI, ARI and sibutramine are more efficacious than MET or reboxetine in reducing weight and metabolic adverse effects (Choi, 2015), showing conflicting results across various studies. As mentioned earlier, the difference in sample size, study duration, adjunctive drugs pharmacokinetics and the patient response might have affected the results. The study by Fernandez and colleagues also suggested genetic involvement in drug response. It was reported that a polymorphism in leptin receptor gene resulted in a blunted response in CLO-treated patients taking MET (Fernandez et al., 2010), raising the possibility of the involvement of genetic factor in drug response. Table 4.1 shows a summary of the adjunctive drugs used in schizophrenic patients who were prescribed CLO and OLA based on double-blind randomised controlled trials data (Mizuno et al., 2014).

Table 4.1. Summary of various add-on drugs used concomitantly with CLO and OLA in schizophrenic patients (modified from (Mizuno et al., 2014).

Adjunctive Drugs	AAPs	Effects on,
Amantadine	OLA	Body weight
Aripiprazole	OLA, CLO	Bodyweight, lipids, glucose metabolism
Atomoxetine	OLA, CLO	Body weight
Famotidine	OLA	Body weight
Fluoxetine	OLA	Body weight
Intranasal insulin	OLA	Body composition
Metformin	OLA, CLO	Body weight, glucose, insulin, lipids, HOMA-IR
Modafinil	OLA, CLO	Body weight
Nizatidine	OLA	bodyweight
Orlistat	OLA, CLO	Body weight
Phenylpropanolamine	CLO	Body weight
Reboxetine	OLA	Body weight
Rosiglitazone	OLA, CLO	Body weight, glucose, insulin, lipids, HOMA-IR
Sibutramine	OLA, CLO	Body weight
Topiramate	OLA, CLO	Effects on body weight and biochemical/metabolic abnormalities
Zonisamide	OLA, CLO	Body weight
Naltrexone	OLA	Body weight
Betahistine	OLA, CLO	Body weight

4.1.1 Selection of drugs and variables

Our data on primary human adipocytes (discussed in Chapter 3) showed deleterious changes in adiponectin secretion and PPARγ protein expression by CLO compared to OLA. Because of this we used CLO as an exemplar in our experiment to see the effect of adjunctive drugs on CLO-treated primary human adipocytes; we used adiponectin secretion and PPARγ protein expression as the key markers of any metabolic effect. Also, both adiponectin and PPARγ are considered as important factors which not only maintain normal function of adipocyte, but also play a role in the improvement of insulin sensitivity in metabolic toxic conditions. Various studies showed the use of numerous drugs as an add-on with CLO. However, we selected drugs (Table 4.2) which showed improved insulin sensitivity in patients with metabolic adverse effects such as obesity and T2DM; or had previously shown promising effects on adiponectin and PPARγ. Clinically relevant therapeutic doses were used for adjunctive drugs while ROSI, a known PPARγ agonist was used as positive control.

Table 4.2: Concomitant drugs used in CLO-treated primary human adipocytes

Adjunctive Drugs with CLO (1µM and 20µM)	Dose
Aripiprazole	0.2µM
Rosiglitazone	10µM
Metformin	10mM
Telmisartan	5µM, 10µM

The sections below provide an overview of the adjunctive drugs used concomitantly with CLO.

4.1.1.1 Metformin

MET is a first-line treatment option in T2DM. Studies with MET in non-psychotic patients with metabolic syndrome showed a reduction in weight gain and improvement in lipid profiles (Rado and von Ammon Cavanaugh, 2016, Chen et al., 2008, Carrizo et al., 2009, Klein et al., 2006). Recently it was reported that schizophrenic patients taking AAPs experience less weight gain if MET is used as an adjunctive drug in the early part of their treatment (Hendrick et al., 2017). MET is a biguanide which has an antiphyperglycaemic effect; it decreases both basal and postprandial glucose. However, it has no direct effect on insulin secretion, so does not cause hypoglycaemia. Various RCT and meta-analysis studies, using MET as an adjunctive drug in AAP-treated schizophrenic patients, showed significant decreases in BMI, waist circumference, triglycerides and glucose levels and increased HDL levels (Zimbron et al., 2016, Carrizo et al., 2009, Chen et al., 2008, Hebrani et al., 2015, Siskind et al., 2016). The proposed mechanism of MET in reversing metabolic parameters may be due to its antagonising effect on glucagon suppressing gluconeogenesis and glycogenolysis, which improves insulin sensitivity (Wiernsperger and Bailey, 1999). It has been reported that CLO-treated patients having the minor allele carrier of the TransMembrane protein (TMEM) 18 (rs6548238) and Glucosamine-6-Phosphate Deaminase (GNPDA2) (rs10938397) showed a significant decrease in body weight after using MET as an adjunctive drug (Chen et al., 2015). TMEM18 is encoded by TMEM18 gene and GWAS studies have shown the association of genetic variants in the vicinity of TMEM18 with obesity (Larder et al., 2017). The previous study showed that TMEM18 is essential for adipogenesis (Bernhard et al., 2013); so it was proposed that MET might reverse metabolic adverse effects through the regulation of adipogenesis (Chen et al., 2013).

4.1.1.2 Aripiprazole

Among all the AAPs, ARI is considered to cause the fewest metabolic adverse effects (Wang et al., 2013). The study by Karunakaran et al showed an average weight loss of 5.1kg in CLO-treated schizophrenic patients using ARI as an adjunctive drug (Karunakaran et al., 2007). Other studies using ARI also showed a significant decrease in weight, BMI and triglycerides levels in AAP-treated schizophrenic patients (Choi, 2015, Wang et al., 2013). However, the study by Henderson et al did not show any change in total serum cholesterol, HDL and LDL-cholesterol after administering ARI concomitantly with OLA-treated patients (Henderson et al., 2009). Choi's meta-analysis reported that on concomitant use of ARI, there was no significant change in PANSS score between groups, but it showed a beneficial effect in the improvement of metabolic adverse effects. The proposed mechanisms of ARI in improving the AAP-mediated adverse metabolic effects might be through improvement in adiponectin secretion. The study by Wang and colleagues showed a significant increase in adiponectin levels in OLA-treated schizophrenic patients given ARI as an adjunctive drug (Wang et al., 2013).

4.1.1.3 Rosiglitazone

ROSI, approved by the FDA in 1999, metabolises glucose and lipids, resulting in improvements in blood glucose levels without producing hypoglycaemia. It activates the PPARγ transcription factor in cells which regulate glucose and lipid metabolism. The DREAM (Diabetes REduction Assessment with ramipril and rosiglitazone Medication) study showed a reversal of insulin resistance to approximately 70% normal by ROSI compared to placebo (Zimbron et al., 2016). However, a double-blind placebo-controlled ROSI trial by Henderson and colleagues showed a non-significant increase in weight gain, BMI and waist circumference with significant improvement

in insulin sensitivity, glucose utilisation and LDL-cholesterol levels (Henderson et al., 2009). ROSI, through its PPARγ agonistic activity, has various physiological actions which include inhibition of pro-inflammatory products, positive effect on insulin sensitivity, decreased inflammation and neural tissue protection from inflammatory products (Yi et al., 2012). However, some studies reported the incidence of myocardial infarction and mortality due to cardiovascular complications caused by ROSI (Nissen and Wolski, 2007, Singh et al., 2007), owing to which its use was withdrawn from the UK and India in 2010 and from NewZealand and South Africa in 2011. However, it is still in use in the USA as FDA initially put restrictions from 2011 to 2013 but later lifted the restrictions after reviewing the 2009 clinical trial results, which showed no association of cardiovascular complications with ROSI (McCarthy, 2013). We have used this drug as a positive control in our experiment as ROSI show beneficial effects on the adipocyte.

4.1.1.4 Telmisartan

Our study was the first of its kind to use TEL in CLO-treated primary human adipocytes. Telmisartan is an angiotensin II type-1 receptor blocker and is used widely in patients as an anti-hypertensive. A study by Derosa and colleagues showed improvement in insulin resistance by TEL in patients with metabolic syndrome (Derosa et al., 2007). Telmisartan acts as a partial agonist on PPARγ and activates it maximally compared to other angiotensin receptor blockers (ARB). Various *in vitro* and preclinical studies also showed improved insulin resistance after using TEL, proposing its action through PPARγ activation (Pushpakom et al., 2017, Benndorf et al., 2006, Foryst-Ludwig et al., 2010, Souza-Mello et al., 2010). TEL has also shown an established effect on adiponectin, a marker for insulin sensitiser, by increasing its concentration. Studies showed that TEL decreased body weight and increased

adiponectin levels in glucose intolerant patients (Makita et al., 2008). A meta-analysis of TEL use in metabolic syndrome patients by Takagi et al reported a significant increase in adiponectin levels and decrease in insulin resistance, depicting TEL efficacy in metabolic adverse effects (Takagi et al., 2013). Pushpakom et al is currently undertaking a clinical trial of TEL in HIV-positive patients to investigate whether TEL can reverse the antiretroviral-induced metabolic toxicity observed in HIV disease (Pushpakom et al., 2015).

4.1.2 Rationale for the work conducted

Cardiometabolic adverse effects, such as obesity, weight gain, diabetes mellitus and metabolic syndrome, are associated with AAPs. It is necessary to find ways to reverse these metabolic adverse effects. Though non-pharmacological approaches (physical activity, caloric restriction and nutritional plan) have been utilised earlier, due to poor rates of uptake, there is a barrier for the effectiveness of these interventions. This has diverted the interest of clinicians to pharmacological interventions to combat the metabolic adverse effects associated with AAPs (Siskind et al., 2016). Various drugs have been used as adjunctive therapy along with AAPs already in the clinic to address AAP-induced metabolic disease. Our rationale was to identify the beneficial effect of selected drugs (MET, ARI, ROSI and TEL) *in vitro* when incubated together with CLO. We also attempted characterisation of potential mechanisms by which they caused reversal of metabolic adverse effects caused by AAPs.

4.1.3 Hypothesis

This part of the PhD tested the hypothesis that adjuvant drugs reverse AAP-induced metabolic adverse effects directly by acting on adipocytes.

4.1.4 Aims and Objectives

1) To characterise potential therapeutic strategies that can reverse AAP-induced metabolic adverse effects in primary human adipocytes.

2) To see the effect of these therapeutic strategies on AAP-mediated changes in adipokine secretion and PPARγ protein expression.

3) To investigate the mechanisms behind the reversal of AAP-induced metabolic adverse effects using primary human adipocytes by assessment of:

a. adiponectin secretion and,

b. PPARγ protein expression.

4.2 Methods

4.2.1 Materials

The materials were similar as described in section 3.2.1. Regarding drugs, CLO, MET and TEL were purchased from Sigma-Aldrich, MO, USA while ARI and ROSI were from Santa Cruz Biotechnology, TX, USA.

4.2.2 Cell culture

Cell culture was performed as described in section 3.2.2. Cells were treated with CLO and different doses of adjuvant drugs starting from day 3 to day 11 (total 5 drug additions). On day 13, 48 hrs after the last drug addition, conditioned media and cell lysates were collected to study adiponectin levels, and PPARγ protein expression respectively. In our experiment, we used 3 donors to check the reproducibility of the results and for statistical purposes.

4.2.3 Measurement of Adiponectin secretion by ELISA

Adiponectin secretion was measured by ELISA as described in section 2.2.5.

4.2.4 Western blotting

Protein isolation, quantitation and western blotting were performed by methodology as described in section 2.2.9.

4.3 Statistical Analysis

All statistical comparisons (3 donors) were made using t-test on StatsDirect software version 2.7.9. Differences were considered significant at $p \leq 0.05$.The result was shown as mean ± SD.

4.4 Results

We have compared the effect of each adjuvant drug (MET, ARI, ROSI and TEL) against both therapeutic (1µM) and high dose (20µM) of CLO.

4.4.1 Effect of adjunctive drugs on adiponectin secretion in CLO-treated primary human adipocytes

We have shown adiponectin levels measured when two different doses (therapeutic dose [1µM] and higher dose [20µM]) of CLO were co-incubated with each adjuvant drug. When co-incubated with 1µM CLO, ARI significantly reversed the CLO-mediated reduction in secretion of adiponectin from the differentiating adipocytes on day 13 (CLO; p=0.04). ROSI, MET and TEL, also showed reversal of CLO-mediated effect on adiponectin secretion, but this was not statistically significant (Fig.4.1A).

For 20µM CLO, all four adjuvant therapeutic strategies showed a trend to reverse CLO-mediated reduction in adiponectin secretion but were not statistically significant (Fig 4.1B).

As discussed in Chapter 3, we believe the lack of statistical significance could once again be an indicator of variability between individual subjects (adipose tissue donors). This would suggest that experiment itself worked fine with respect to individual drug effects; however, because of the variability between donors, we observed marked differences in the magnitude of the effect (Fig.4.1C-D).

Fig 4.1 A

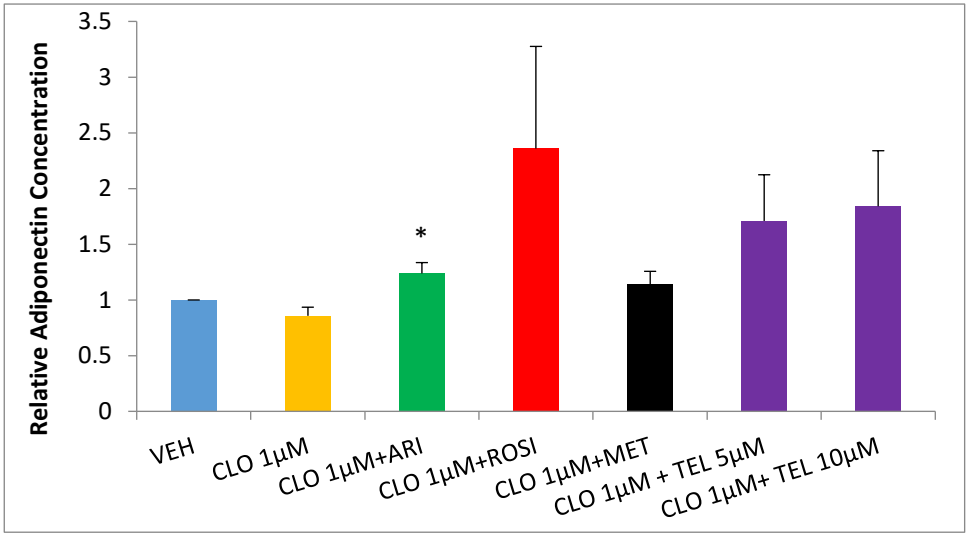


Fig 4.1 B

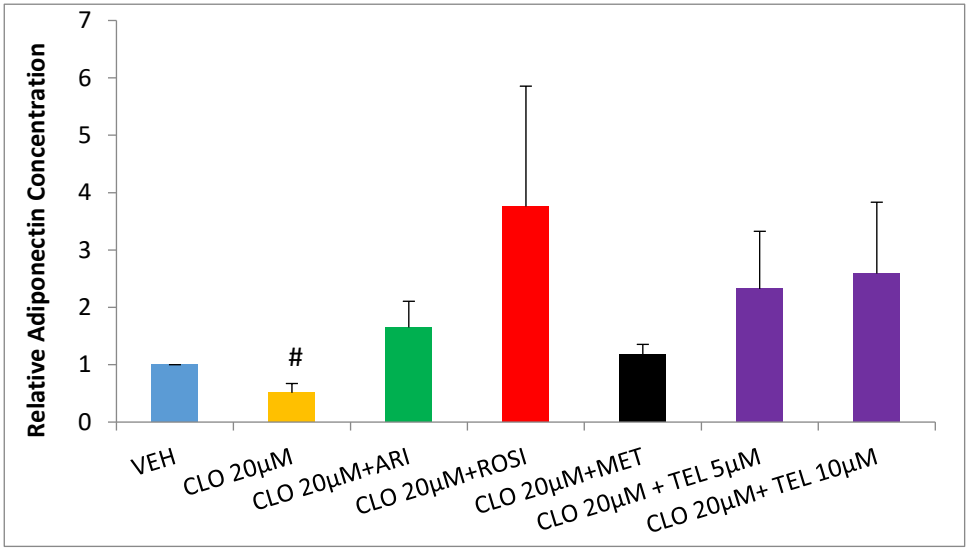


Figure 4.1: Effect of co-incubation of various adjuvant drugs (ARI, ROSI, MET, TEL) on A) CLO (1µM) and B) CLO 20µM-mediated reduction in secreted adiponectin in differentiating primary human adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as mean ± SD; $p \leq 0.05$. * 1µM CLO vs 1µM CLO+ARI, # VEH vs 20µM CLO.

Fig 4.1 C

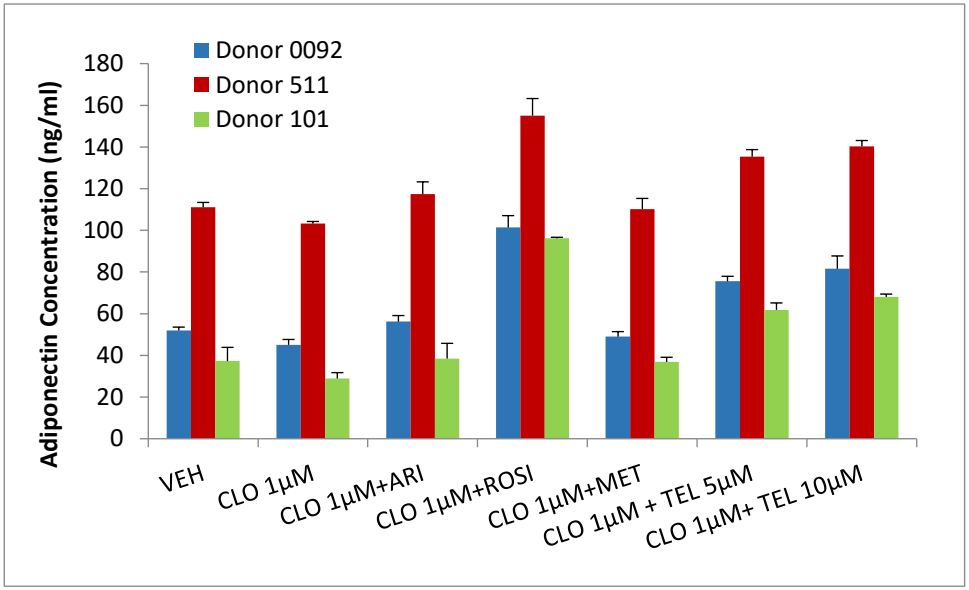


Fig 4.1 D

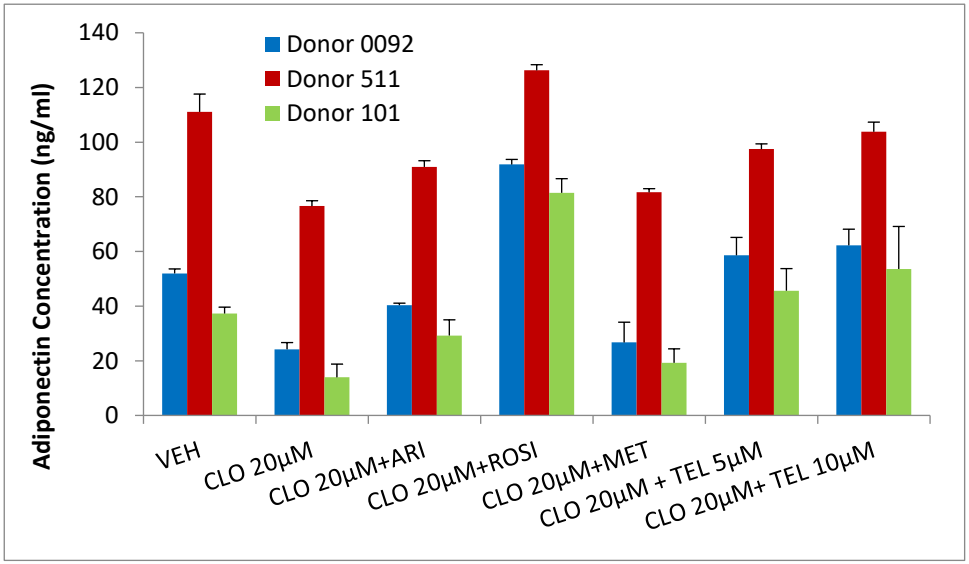


Figure 4.1: C) Change in adiponectin secretion observed in individual donors (n=3) with 1μM CLO alone and when co-incubated with adjuvant drugs (ARI, ROSI, MET, TEL); D) Change in adiponectin secretion observed in individual donors (n=3) with 20μM CLO alone and when co-incubated with adjuvant drugs.

4.4.2 Effect of adjunctive drugs on PPARγ protein expression in CLO-treated primary human adipocytes

Our data showed that therapeutic dose of CLO (1μM) cause significantly increased (p=0.04) PPARγ expression. The higher dose of CLO (20μM) showed a trend to increase PPARγ expression but was statistically non-significant (Fig. 4.2A).

We have shown PPARγ expression levels when therapeutic and higher dose of CLO were co-incubated with each adjuvant drug. When co-incubated with 1μM CLO, ARI, MET and TEL (5μM) resulted in a trend to further increase PPARγ expression compared to 1μM CLO alone; however this was not statistically significant. TEL (10μM) co-incubation with 1μM CLO resulted in significantly decreased expression of PPARγ as compared to 1μM CLO alone (p=0.03). On the other hand, ROSI when co-incubated with 1μM CLO showed a further significant increase in PPARγ expression (p=0.01) (Fig 4.2B).

For 20μM CLO, only ROSI showed a further increase in expression which was statistically significant (p=0.01). All other drugs (ARI, MET and TEL, 5μM) did not affect the 20μM CLO-mediated increase in PPARγ expression. However co-incubation of 10μM TEL with 20μM CLO showed a significant decrease in the expression of PPARγ as compared to 20μM CLO alone (p=0.03) (Fig. 4.2C).

The lack of statistical significance could be an indicator of variability between individual subjects (adipose tissue donors). This would suggest that experiment itself worked fine with respect to individual drug effects; however, because of the variability between donors, we observed marked differences in the magnitude of the effect (Fig. 4.2D-E).

Fig 4.2 A

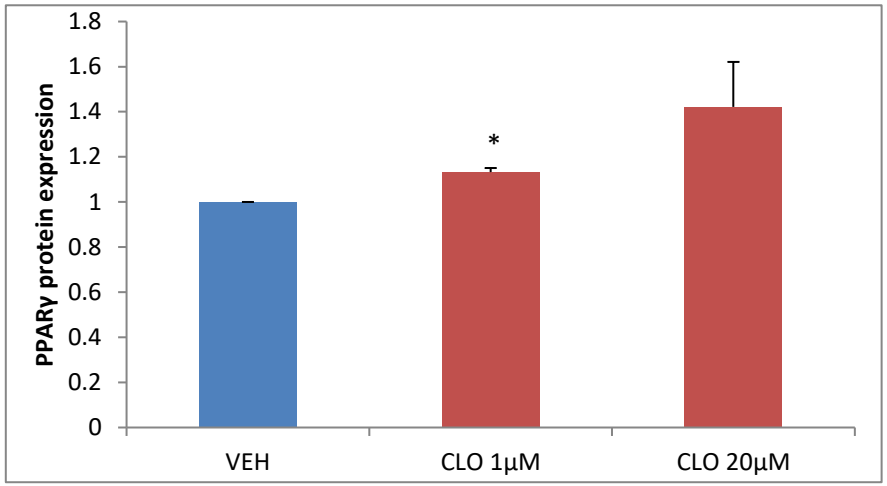


Fig 4.2 B: 1µM CLO plus Adjuvant drugs

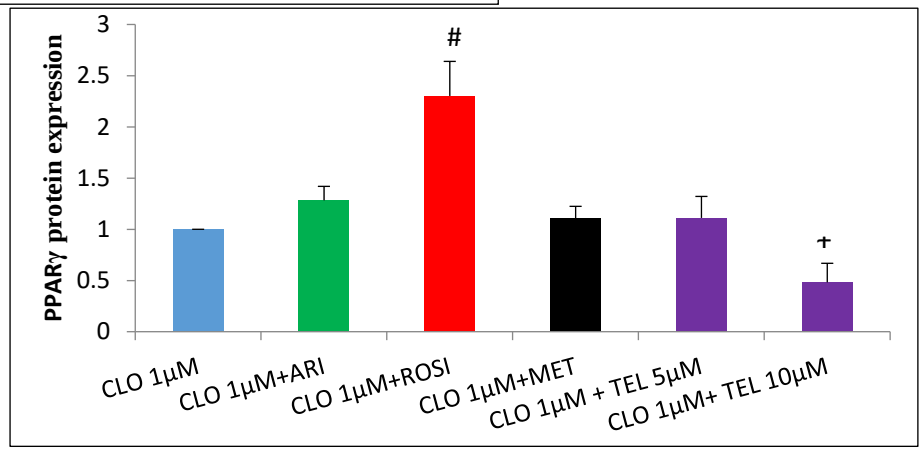
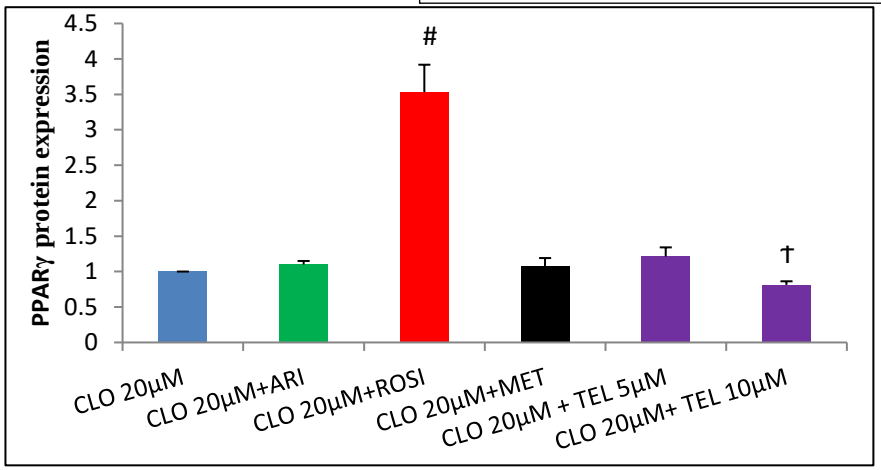


Fig 4.2 C: 20µM CLO plus Adjuvant drugs



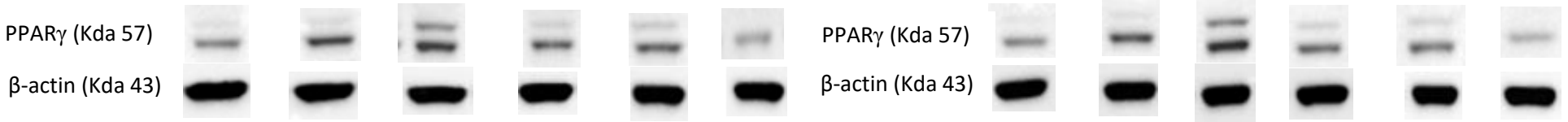


Figure 4.2: A) PPARγ protein expression showing the effect of various concentrations of CLO on differentiated adipocytes. Effect of co-incubation of various adjuvant drugs (ARI, ROSI, MET, TEL) on B) CLO (1µM) and C) CLO 20µM-mediated increase in PPARγ protein expression in differentiating primary human adipocytes. All experiments were done in adipocytes cultured from 3 individual donors. Data was shown as mean ± SD; $p \leq 0.05$. * VEH vs 1µM CLO, #1µM/20µM CLO vs ROSI, † 1µM/20µM CLO vs TEL 10µM.

Fig 4.2 D

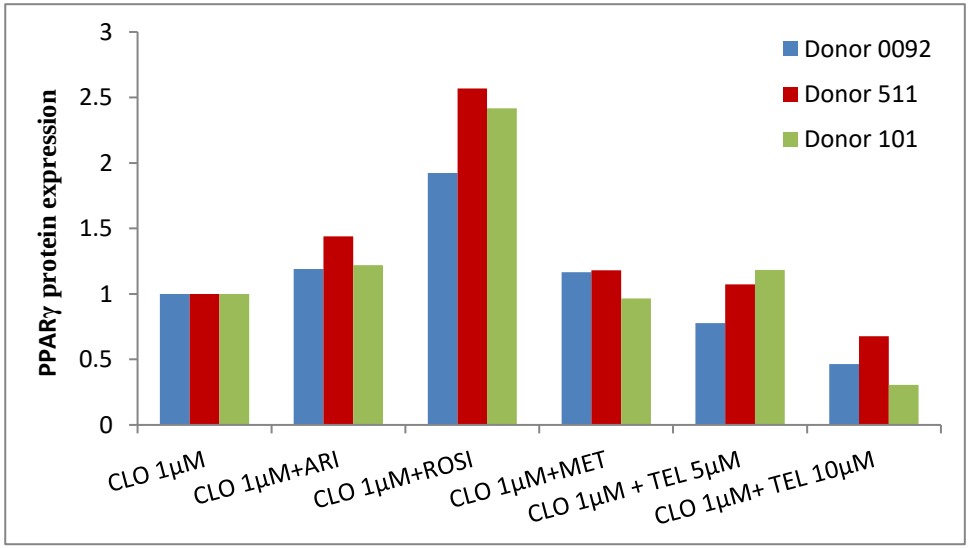


Fig 4.2 E

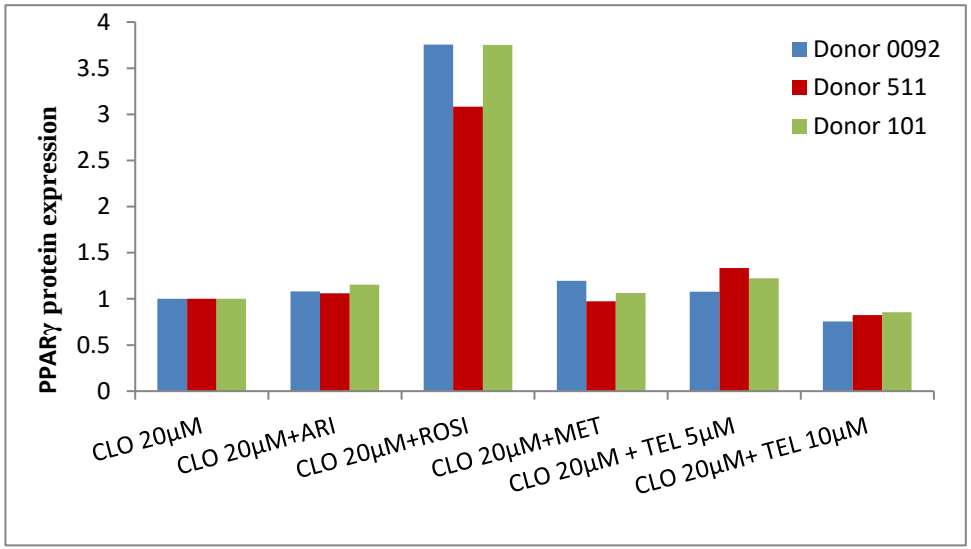


Figure 4.2: D) Change in PPARγ protein expression observed in individual donors (n=3) with 1μM CLO alone and when co-incubated with adjuvant drugs (ARI, ROSI, MET, TEL); D) Change in PPARγ protein expression observed in individual donors (n=3) with 20μM CLO alone and when co-incubated with adjuvant drugs.

4.5 Discussion

Various clinical trials that investigated the use of adjuvant drugs with AAPs have showed efficacy in the reversal of AAP-induced metabolic adverse effects. Some studies proposed MET to be used as first line adjuvant therapy to reverse AAP-induced weight gain and metabolic adverse effects, while others proposed TOPI and ARI as preferred adjunctive drugs (Whitney et al., 2015, Mizuno et al., 2014, Maayan et al., 2010). The differences between these individual trials may be due to differences in the study design, duration and effect of drugs or related to the pharmacokinetic or pharmacodynamic properties of adjuvant drugs and their interactions with antipsychotic drugs (Cipriani et al., 2009, Zimbron et al., 2016, Choi, 2015). We used 3 adjunctive drugs (ARI, MET, TEL) plus one drug, ROSI, as a positive control, to see their effects on CLO-induced changes in adiponectin secretion and PPARγ expression as it has been suggested that these parameters play an important role in adipogenesis and in regulating insulin sensitivity. The adjunctive drugs used in our experiment show beneficial metabolic effects and have previously shown a promising effect on insulin sensitivity and other important metabolic parameters such as weight gain, lipids and adipocytokines. However, the mechanistic effects of these drugs on adiponectin secretion and PPARγ expression when used alongside AAPs in an *in vitro* primary human adipocyte model have not been reported before.

Our *in vitro* studies showed ARI significantly reversed CLO-mediated reduction in adiponectin secretion. ARI was effective when used along with both therapeutically relevant and a higher concentration of CLO. Our result is consistent with the study by Wang et al (Wang et al., 2013) showing increase in adiponectin secretion after giving ARI as an adjunctive drug to schizophrenic patients using AAPs. This suggests the mechanistic role of ARI in reversing metabolic adverse effects through adiponectin. Adiponectin acts as an insulin sensitiser and its levels have been documented to

decrease in patients having insulin resistance in conditions like obesity or T2DM (Jin et al., 2008). It has been postulated that adiponectin activates the AMPK kinase pathway which activates a series of reactions leading to increased fatty acid oxidation, decreased gluconeogenesis in the liver and decreased plasma glucose levels, ultimately resulting in improved peripheral insulin sensitivity (Gil-Campos et al., 2004). This suggests that ARI reverses insulin resistance in metabolic toxic conditions by increasing the secretion of adiponectin, which may thereby improve insulin sensitivity. However, most of the adjunct studies of CLO and ARI were focused on other metabolic parameters and weight gain. Henderson and colleagues reported a significant decrease in body weight, BMI, serum cholesterol and serum triglycerides on concomitant use of ARI with CLO in schizophrenic patients (Henderson et al., 2006). Fleischhacker et al also reported reduction in body weight, BMI and waist circumference in schizophrenic patients; though there was no improvement in PANSS scores, but the clinical global impression (CGI) scale showed some improvement in the treatment group (Fleischhacker et al., 2010). However, there are also some studies which showed improvement in PANSS score after administering ARI concomitantly with CLO in schizophrenic patients (Galling et al., 2017, Srisurapanont et al., 2015). This may suggest the involvement of multiple factors other than just adiponectin in reversing the AAP-induced metabolic adverse effects. Along with improvement in schizophrenic symptoms, there has been a report of akathisia as an adverse effect when ARI was given concomitantly with CLO, therefore monitoring is required when both drugs are given together (Choi, 2015). Although we observed a reversal in CLO-mediated effect on adiponectin secretion, our data also showed high variability between the 3 donors (this resulted in ARI's effect on 20μM CLO to be statistically non-significant). This is because of using only 3 biological replicates due to

limitations in the availability of adipocytes from donors, and managing a chronic toxicity experiment in large number of replicates. However, all our 3 biological replicates showed a similar trend in adiponectin secretion (increased secretion) when analysed individually. Our data also showed a trend to further increase PPARγ expression by ARI when used concomitantly with therapeutic doses of CLO, suggesting a mechanistic role of PPARγ in a reversal of metabolic adverse effects. Both the increase in adiponectin and PPARγ may suggest that ARI activates PPARγ, which is a regulator of adiponectin, resulting in an improvement in insulin sensitivity. This shows a strong interaction between PPARγ and adiponectin. As PPARγ is considered the master regulator of adipogenesis, ARI not only maintains adipogenesis but also improves insulin sensitivity through adiponectin. However, ARI did not show any change in PPARγ expression when co-incubated with high doses of CLO; it did show a trend to increase adiponectin secretion but that effect was not significant. This might be because the concentration we used for ARI may not be sufficient to counter the effects of high CLO dose (20μM). The other proposed mechanism in reversing AAPs induced metabolic adverse effects by ARI could be by activation of P13K/Akt pathway. This pathway maintains cellular growth and metabolism, leading to an improvement in insulin sensitivity. Disturbance in this pathway leads to metabolic disorder related adverse effects. It was argued that this pathway augmented insulin through GLUT4 and AAPs targeted this P13K/Akt pathway, affecting GLUT 4 activation. It was proposed that ARI activates this pathway through activation of the D2 receptor as the previous study showed that D2 receptor agonist drug bromocriptine regulates the P13K/Akt pathway (Deng et al., 2010). We have not investigated the effect of ARI on this pathway or its mediators; this could be an area for future research.

In our experiment, ROSI was used as positive control and it showed a trend to reverse CLO-mediated effect on adiponectin concentration in CLO-treated cells. It also showed a significant increase in PPARγ expression when co-incubated with both doses of CLO. Various *in vitro* preclinical and clinical studies have shown an increase in adiponectin secretion on administering ROSI alone, which proposed the idea that ROSI improved insulin sensitivity by increasing adiponectin secretion (Yamauchi et al., 2001, Maeda et al., 2002, Combs et al., 2002, Hirose et al., 2002, Leung et al., 2016). It was also found that ROSI produced an insulin-sensitising effect through activation of PPARγ. It was then proposed that PPARγ might produce its effect through activation of adiponectin, increasing its levels leading to improvement in insulin sensitivity (Yu et al., 2002). PPARγ is mostly expressed in adipose tissue and insulin sensitivity improvement takes place mainly in skeletal muscle where PPARγ is less expressed. This postulated that ROSI modulates the signalling between adipocytes and muscle cells and may involve adiponectin to improve insulin sensitivity (Sharma et al., 2006). Various clinical trials of AAPs with ROSI showed increased weight gain but a decrease in HOMA-IR and small LDL particle number (Baptista et al., 2009, Mizuno et al., 2014). It was proposed that this small LDL particle number was an important risk factor in metabolic syndrome, leading to cardiovascular diseases (Friedlander et al., 2000, El Harchaoui et al., 2007), so lowering of this particle would decrease the risk factor in metabolic syndrome. However the weight gain caused by ROSI could be of concern when used concomitantly with AAPs. It should also be noted that due to cardiovascular complications caused, the clinical use of ROSI has been restricted in many countries.

Our MET data showed a trend to increase in adiponectin secretion in CLO-treated cells, but the result was non-significant. This coincided with the results from studies

which showed an increase in adiponectin secretion, both *in vitro* using primary human adipocytes and *in vivo* in Zucker diabetic fatty rats (Schmid et al., 2013, Zulian et al., 2011). This has been replicated in another preclinical study which also showed increased adiponectin secretion after using MET (Saad et al., 2015). Meta-analysis of MET along with AAPs showed reduction in weight, BMI, weight circumference, HOMA-IR, improvement in fasting glucose and triglycerides (Liu et al., 2015, Ehret et al., 2010, Rado and von Ammon Cavanaugh, 2016, Baptista et al., 2006, Wu et al., 2008, Praharaj et al., 2011). Our data with MET also showed a trend to increase PPARγ expression in cells treated with 1μM CLO, suggesting that MET might activate PPARγ leading to activation of adiponectin, in turn leading to increased insulin sensitivity. It was also proposed that MET may activate AMP-activated protein kinase, which ultimately improved insulin sensitivity (Pernicova and Korbonits, 2014). This suggests that MET may reverse metabolic toxic effects through multiple mechanisms. However, MET showed no change in PPARγ expression in adipocytes treated with high dose CLO (20uM). This could be related to the concentration as observed with ARI earlier.

Our TEL data showed a trend to increase adiponectin secretion by both doses. PPARγ expression also showed a trend to increase by 5μM dose of TEL, suggesting interrelation between adiponectin and PPARγ. Nagel et al reported interaction between adiponectin and PPARγ and proposed that TEL induces adiponectin through PPARγ activation (Nagel et al., 2006). A study by Benndorf and colleagues also reported improvement in insulin sensitivity through activation of PPARγ by TEL (Benndorf et al., 2006). Yamashita and colleagues studied the effect of concomitant use of TEL in OLA treated patients and it was proposed that TEL might improve insulin sensitivity by affecting adipokines levels, especially adiponectin (Yamashita et

al., 2008). It has been reported that ARB also acts as PPARγ agonist, which on activation increases the expression of PPARγ target genes which regulate lipid and carbohydrate metabolism (Iwai et al., 2007, Benson et al., 2004, Kurtz, 2006, Schupp et al., 2004). Our data showed that TEL improves insulin sensitivity through increase secretion of adiponectin suggesting its direct effect on adiponectin. However, TEL (10µM) but not 5µM TEL, showed decreased PPARγ expression in both doses of CLO treated cells, owing to TEL's partial agonistic activity which showed that increase dose of TEL (10µM TEL) have more antagonistic effect instead of agonistic effect leading to decreased PPARγ expression. However, more research is required, especially on drug interaction between CLO and TEL to explore the exact mechanism. Our data on co-incubation of adjunctive drugs with AAPs suggest they might be able to reverse the metabolic adverse effects caused by AAPs through improvement in adiponectin secretion or activation of PPARγ or a combination of both. The role of adiponectin as insulin sensitising and anti-atherogenic protein has been well documented and it has been established that obese patients have lower adiponectin levels than non-obese (Yang et al., 2004). Maeda and colleagues showed the knockdown of adiponectin in mice resulted in hyperglycaemia and hyperinsulinemia. It has also been documented that the PPARγ agonist up-regulate serum adiponectin (Maeda et al., 2002). A study by Yang and colleagues on rats showed a positive correlation between PPARγ agonist and increased adiponectin levels, which resulted in improved insulin sensitivity and a lipid-lowering effect (Yang et al., 2004). Studies also proposed that PPARγ agonist may stimulate the production of adiponectin. This adiponectin then activates AMPK, which increases the fatty acid oxidation. This leads to the activation of cascade which ends with the uptake of glucose and lactose production by muscle cells. This results in reduced glucose levels and increased

sensitivity in liver and muscle (Guan et al., 2002, Combs et al., 2002, Gil-Campos et al., 2004). Apart from the activation of adiponectin by PPARγ, studies proposed that PPARγ also affects the insulin-signalling cascade and it does this by regulation of expression or phosphorylation of signalling molecules. Insulin, on binding to its tyrosine kinase receptors, starts a signalling cascade which leads to the activation of insulin receptor substrate and phosphatidylinositol-3-kinase (PI-3 kinase) and other downstream kinases which regulate processes like glucose uptake, lipid metabolism, differentiation and gene transcription regulation (Jiang et al., 2004, Miyazaki et al., 2003, Jiang et al., 2002). PPARγ also decreases FFA levels by inhibiting adipocyte lipolysis which modulates insulin sensitivity (Bays et al., 2004).

In summary, adjunctive drugs (therapeutic concentration) effect on CLO-mediated adiponectin secretion and PPARγ expression in *in vitro* primary human adipocyte model have not previously been studied. All these 4 drugs have had an effect on adiponectin and PPARγ which may work together or independently to improve insulin sensitivity and can be a potential therapeutic target to reverse AAPs induced metabolic adverse effects. Our data demonstrated high inter-individual variability between the 3 adipocyte donors which limits statistical significance of the results, and the conclusions that can be drawn. In future studies a large number of donors may decrease the variability. Figure 4.3 showed the proposed mechanisms of adjunctive drugs through adiponectin and PPARγ.

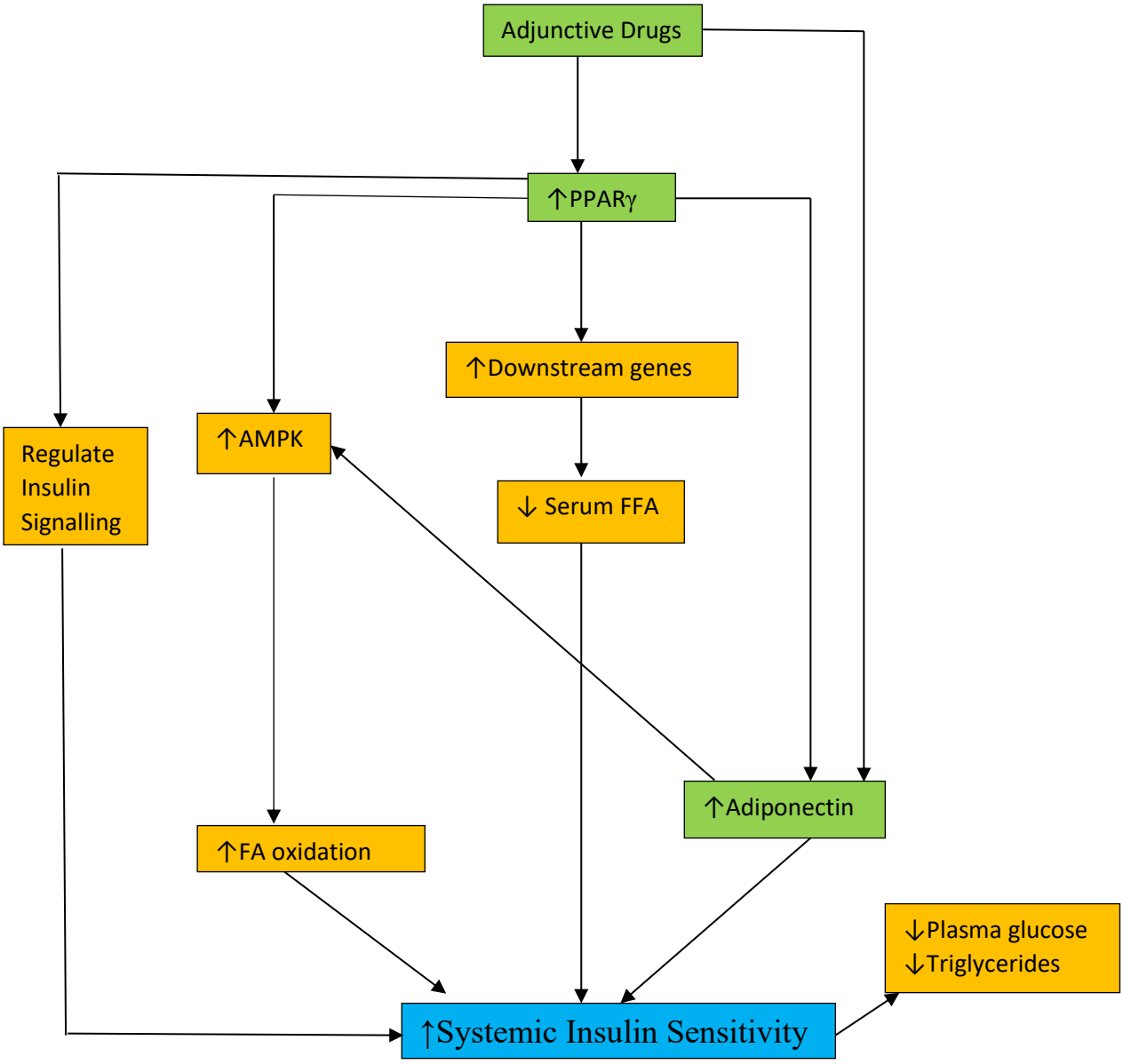


Figure 4.3 Proposed mechanisms of adjunctive drugs through PPARγ and Adiponectin

Chapter 5

Effect of Atypical antipsychotics on human adipocyte lipidome

5.1 Lipidomics

Lipidomics is defined as a global study of molecular lipids in a biological system which encompasses its analysis and characterisation along with interaction with other lipid classes and partners like proteins (Castro-Perez et al., 2010, Wenk, 2005). Lipidome encompasses various lipid species present in a cell without which the cell cannot perform its physiological actions (Serhan et al., 2006). Lipidomics is considered to be a subgroup of metabolomics as shown in Figure 5.1.

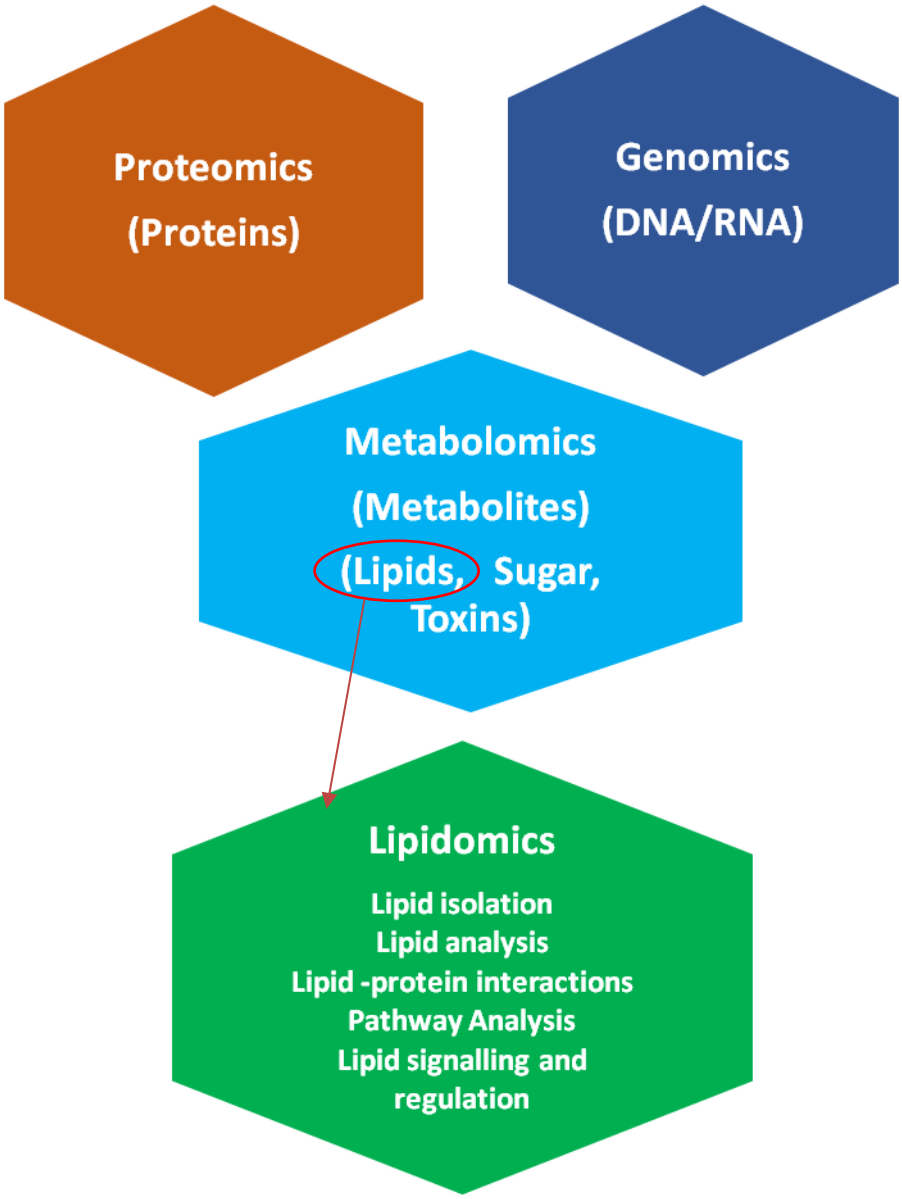


Figure 5.1 Analysis of cells through global profiling (genomics, proteomics, and metabolomics). Lipidomics is a subgroup within the field of metabolomics.

Lipidomics could be helpful in providing information on disease mechanisms. Lipids control many biological functions including regulatory, structural and bioenergetic functions. The key function of lipids includes energy storage, cell signalling, protein trafficking and membrane anchoring. Various studies have shown that disturbances in lipid metabolism can result in the onset and progression of many human diseases including obesity, cancer, Alzheimer's disease and atherosclerosis (Cutler et al., 2004, Lusis, 2000, Menendez and Lupu, 2007, Shi and Burn, 2004).

5.1.1 Classification of Lipids

Lipids are hydrophobic substances which are soluble in organic solvents. Lipids are classified into 8 categories. These are further subclassified based on the attachment of hydrophilic and hydrophobic elements (Fahy et al., 2009). Table 5.1 shows the classification and subclasses of lipids.

Table 5.1: Classification of lipids (LIPID MAPS Database)

Category	Abbreviation	Subclasses
Fatty acyls	FA	Fatty acids and conjugates, Eicosanoids
Glycerolipids	GL	Diacylglycerol (DAG), Triacylglycerol (TAG)
Glycerophospholipids	GPLs	Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidic acid, Phosphatidylserine, phosphatidylglycerol phosphatidylinositol
Sphingolipids	SL	Ceramides, sphingomyelin, sphingosine, Dihydroceramides, dihydrosphingosine, ganglioside
Sterol lipids	ST	Cholesterol, cholesteryl ester
Prenol lipids	PL	Isoprenoids
Saccharolipids	SP	Acylaminosugars
Polyketides	PK	Macrolide polyketides

The cell membrane, which acts as a communication border between the interior and exterior of the cells, is composed of a phospholipid bilayer along with proteins and other lipids such as sterols and glycolipids. These membrane lipids play an important role in cell proliferation, signal transduction pathways, apoptosis and movement along the membrane. Glycerophospholipids (GPLs), sterol, and sphingolipids (SL) constitute the main bioactive lipids in a biological membrane of mammalian cells (Bou Khalil et al., 2010).This chapter will focus more on the SL subclass named ceramides (Cer).

5.1.1.1 Sphingolipids (SL)

SL are abundant in many living organisms including humans, plants, and yeast. SL, which contain a backbone of SL long-chain base (sphinganine and sphingosine), not only maintain the integrity of cell membranes but also regulate cell growth, differentiation, apoptosis, chemotaxis and signalling pathways (Haynes et al., 2009). The main classes of SL include Cer, dihydroceramides (DhCer), sphingomyelin, dihydrosphingomyelin, sphingosine, sphinganine, galactosyl ceramide and sphinganine-1-phosphate. Their structures are given in Figure 5.2. Sphingomyelin, which is comprised of Cer moiety, is linked to the phosphocholine group. Sphingomyelin's function is the assembly of signalling molecules and regulation of trafficking of membrane proteins and various cellular processes (Lahiri and Futerman, 2007). Cer, an important SL, acts as an apoptotic mediator while sphingosine-1-phosphate regulates calcium homeostasis, cell growth, immune cells migration and suppression of apoptosis (Spiegel and Milstien, 2003, Lahiri and Futerman, 2007).

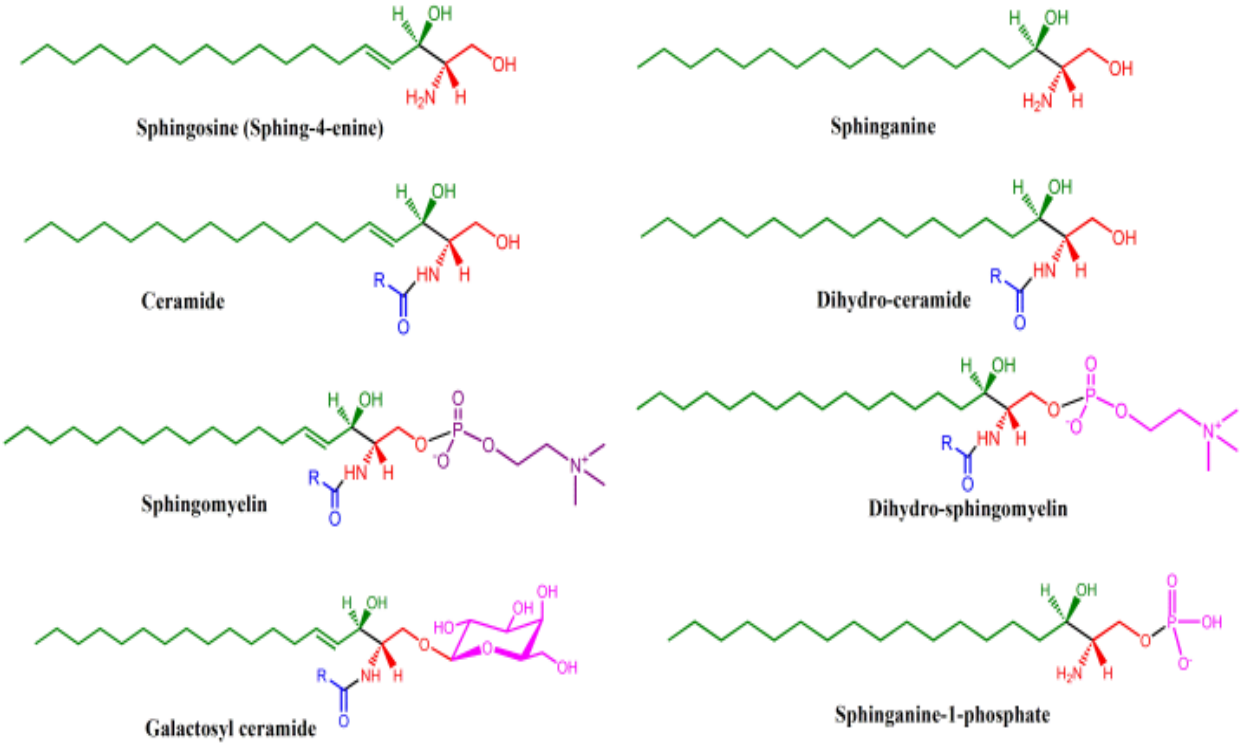


Figure 5.2. Structure of Sphingolipid classes (adapted from Yang Yu, 2012)

5.1.2 Analytical techniques used for lipidome profiling

Lipidomic analysis has been a challenging task due to the high diversity of lipids. Initial studies have been focused on the analysis of individual lipid classes through thin layer chromatography (TLC). However, there was rapid progress in this field after the introduction of Mass Spectrometry (MS)-based techniques. Untargeted approaches for global analysis of lipids, and targeted analysis for specific lipid classes, have been applied to discover biomarkers for diseases, such as cardiovascular disease, cancer and metabolic diseases (Markgraf et al., 2016). Below, a brief description of various analytical techniques used to study lipids is given.

5.1.2.1 Chromatography

Liquid chromatography (LC) techniques have successfully shown their usefulness in the analysis of complex lipids and their species. TLC, one of earlier developments of LC, also showed promising results in the separation and analysis of lipids. TLC is developed from paper chromatography and is composed of a thin layer of stationary phases such as silica or cellulose. Numerous combinations of aqueous stationary and organic mobile phases resulted in the separation of various classes of lipids; however, low resolution and the sensitivity of TLC mean that many important lipids remain undetected (Houjou et al., 2005, Hermansson et al., 2005). Another technology used was solid phase extraction (SPE). This technology is used to separate lipid complexes into its classes including fatty acids, cholesterol ester, acyl glycerol, and phospholipids. However, both techniques were replaced by high-performance liquid chromatography (HPLC) which gained attention due to its selective and efficient properties. In HPLC, the lipids are separated through normal-phase or reverse phase columns. In normal-phase, lipids are separated based on the polar head group while, in reverse-phase, separation is due to polarity, alkyl chain saturation, and chain length. Gas chromatography (GC) is another chromatographic technique widely used for the detection of fatty acids; however, this technology shows more efficient and selective results when combined with MS (Lesnefsky et al., 2000).

5.1.2.2 Mass Spectrometry

MS measures both the mass-to-charge ratio of molecules and its intensity, thus it acts as an important technique to separate and quantify various lipid species. However, the development of Electrospray Isonisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) has expanded the range of lipids analysed by MS in

the samples. Various lipidomic studies showed promising data after using these techniques (Pulfer and Murphy, 2003, Han and Gross, 2001, Griffiths, 2003). The ESI-MS technology has been used for analysis of phospholipids, sphingolipids, and acylglycerol (Haynes et al., 2009, Brugger et al., 1997). Multidimensional MS-based shotgun lipidomics is another approach used for the identification of fatty acid isomers and various individual lipid species (Han, 2016).

5.1.2.3 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) is a powerful technology which provides information on the molecular structure of purified lipids (^{1}H-NMR and ^{13}C-NMR). It also provides information on the structure and dynamics of lipid membranes. However, the lipid restricted movement in bilayers or lipoproteins leads to poor resolution. Also, NMR techniques have moderate sensitivity compared to MS (Wenk, 2005). Table 5.2 summarise the technologies used for lipidomic research.

Table 5.2: Technologies for lipidomic research

Technology	**Lipid class covered**	**Advantages**	**Disadvantages**
Chromatography			
Thin Layer Chromatography	Most lipids	Technically easy, minor instrumental investment	Low resolution and sensitivity

Gas Chromatography	Triglycerides, Fatty acids, and sterols	Mainly used for fatty acids	Require volatile compounds
High-performance liquid chromatography	Many lipids	Quantitative, easy automation	Medium sensitivities
Mass Spectrometry MALDI	Many lipids	Contaminants controlled technique	Matrix background, ionization suppression
ESI	Polar compounds	High resolution and sensitivity, easy automation	Ionization suppression
NMR ^{1}H	All lipids	Direct measurement, excellent technique for structural analysis of purified compounds	Low sensitivity
^{31}P	Phospholipids	Quantitative, direct measurement	Low sensitivity
Biochemistry Reactive lipids	Few	Identification of lipid-binding proteins	a limited number of probes, specificity
Lipid antibodies	Very few	Cell biology study	Antibody specificity
Soluble lipids in assays	Many lipids	Quantitative binding studies	Optimization challenging
Immobilized lipids in assays	Many lipids	Identification of ligand-lipid interactions	Automation generally difficult

ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; NMR nuclear magnetic resonance

5.1.3 Data Processing Software for Lipid Analysis

Analytical instruments produce a huge amount of data to process. There are many types of data-processing software available for lipidomic analysis. The steps in the lipidomic analysis include peak detection, lipid identification, isotope correction, response correction, and quantification. However, the same software or data processing tools cannot be applied for different data formats as every vendor has different set ups for the detection of lipids. The examples include Lipid Qualitative/Quantitative Analysis (LipidQA) software which identifies and quantifies complex lipids from the data coming from the TSQ-7000 triple stage quadrupole mass spectrometer (Thermo-Finnigan) and the Q-TOF hybrid quadrupole time-of-flight (Waters-Micromass). The fatty acid analysis tool (FAAT) is specifically for data using mycobacteria species using Fourier transform mass spectrometry. The other types of software used are Lipid Profiler, LipidInspector and cognoscitive-contrast-angle algorithm and database (COCAD). MZmine software is open source software for LC/MS-based samples particularly focusing on lipidomics analysis from the UPLC/MS experimental setup (Ejsing et al., 2009, Leavell and Leary, 2006, Katajamaa et al., 2006).

5.1.4 Lipid Databases

Several databases exist which provide detailed information on lipids such as LIPID MAPS, Lipid Bank, Cyber lipids, and LIPIDAT. The most commonly used database is LIPID MAPS which represents the lipid compounds with a unique 12-digit identifier. The LIPID MAPS Proteome Database (LMPD) provides information on lipid-associated protein sequences and annotations while the LIPID MAPS Structure Database (LMSD) focuses on lipid structure as well as other related information with

respect to the classification scheme recommended by the LIPID MAPS consortium. LMSD is also a useful tool for mass spectrometry data. Providing information on classification, nomenclature and chemical representation, LIPID MAPS is considered a standard reference tool for the construction of lipid databases and management of lipidomics data. Lipid databases also provide information on lipid pathways. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database provides information on metabolic pathways including lipid pathways. SphinGOMAP offers information about sphingolipids and glycosphingolipids lipid species. The LIPID MAPS biopathways workbench helps to display, edit and analyse lipids pathways (Yetukuri et al., 2007, Cotter et al., 2006, Fahy et al., 2009, Kanehisa et al., 2004). The summary of various lipid databases is given in Table 5.3.

Table 5.3. Lipid databases and their characteristic features

Lipid Database	Description
LIPID MAPS (www.lipidmaps.org)	Provide information on lipid classification and structure, contain lipid-associated protein data and develop tools for identifying lipids
CyberLipids (www.cyberlipid.org)	Provide collection of updated scientific knowledge on all aspects of lipids and contain updated bibliography related to lipid biology
LIPIDAT https://www.crcpress.com/LIPIDAT-/Caffrey/p/book/9780849389245	Compilation of thermodynamic data and bibliographic information on lipids. It also includes the effect of pH, protein, drugs, salt, and metal ion concentration on these thermodynamic values.
LIPID BANK (www.lipidbank.jp)	Provide information on chemical structure, physical and chemical properties, biological activities and metabolism of lipids. Also, spectral information from instruments like ultraviolet, infrared spectrometry, nuclear magnetic resonance, mass spectrometry, thin layer chromatography and liquid chromatography can be obtained
KEGG lipids (http://www.genome.jp/kegg-bin/get_htext?br08002.keg)	Give information on lipid pathways and associated information such as name, structure, mass, formula, chemical reactions. Also, provide information on external links to other databases
THE LIPID LIBRARY (http://www.lipidlibrary.co.uk/)	Provide information on various classes of lipids and their analysis both in mass spectrometry and chromatography
sphinGOMAP (http://www.sphingomap.org/)	Offers information on sphingo and glycosphingolipids

5.1.5 Lipid Nomenclature

Lipids are named in two different ways: 1) systematic name and, 2) common or trivial name. Common name includes abbreviations which show acyl or alkyl chain in

acylglycerols, sphingolipids and GPLs. The guidelines for lipid systematic names have been recommended by International Union of Pure and Applied Chemists and the International Union of Biochemistry and Molecular Biology (IUPAC-IUBMB) Commission on Biochemical Nomenclature (http://www.chem.qmul.ac.uk/iupac/).

5.1.5.1 Sphingolipid nomenclature

Sphingolipid structure have (i) amine-containing lipid backbones (sphingoid bases: sphingosine, sphinganine and phytosphingosine) with amide-linked fatty acids and a (ii) head group, which range from a simple hydroxyl in Cer to highly complex glycoconjugates.

5.1.5.1.1 Sphingoid bases

The chemical name of sphingoid base (18-carbon), as shown in Figure 5.5 is (2S,3R,4E)-2-aminooctadec-4-ene-1,3-diol,which is referred as sphingosine, a major species found in mammalian sphingolipids. It is now called as (E)-sphing-4-enine by the IUPAC nomenclature. Sphingoid base nomenclature provides the number of hydroxyl groups: d for the two (di-) hydroxyls of sphingosine and sphinganine, and t (tri-) for the additional hydroxyl in 4-hydroxysphinganine; followed by the chain length (typically 18 carbons); and the number of double bonds (0, 1 or 2); therefore, sphingosine is abbreviated d18:1 (Pruett et al., 2008).

5.1.5.1.2 Nomenclature of ceramides

In ceramides, most of the sphingoid bases are N-acylated with long-chain fatty acids (Fig. 5.3). These fatty acids vary in chain length (14 to 36 carbon atoms), and by the presence or absence of a hydroxyl group on the carbon atom. The recent notation denotes “ceramide” for N-acylsphingosines; dihydroceramides for N-

acylsphinganines; and, 4-hydroxyceramides and phytoceramides for N-acyl-4-hydroxysphinganines. The fatty acyl chain length can also be shown as a prefix, such as C18-Cer for N-stearoylsphingosine. When the sphingoid base is abbreviated, the fatty acyl chain is presented after the sphingoid base; for example, N-stearoylsphingosine or C18-Cer is abbreviated d18:1/18:0 (Zheng et al., 2006).

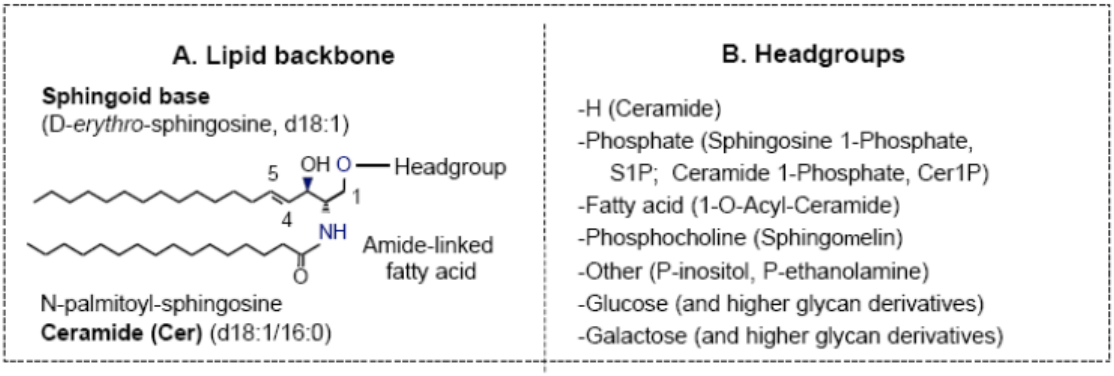


Figure 5.3 Structure and Nomenclature of sphingolipids (adapted from Hirabayashi, 2006)

Following table (Table 5.4) showed the systematic and common name of Cer18-Cer species which has been repeated throughout this chapter.

Table 5.4 Common and Systematic name of Cer18-Cer (Adapted from www.lipidmaps.org)

Common Name	Systematic name
d18:1/16:0	N-(hexadecanoyl)-sphing-4-enine or N-Palmitoyl-sphingosine
d18:1/16:1	N-(9Z-hexadecenoyl)-sphing-4-enine-1-phosphocholine
d18:1/17:0	N-(heptadecanoyl)-sphing-4-enine

d18:1/18:0	N-(octadecanoyl)-sphing-4-enine
d18:1/18:1	N-(9Z-octadecenoyl)-sphing-4-enine
d18:1/19:0	N-(nonadecanoyl)-sphing-4-enine
d18:1/20:0	N-(eicosanoyl)-sphing-4-enine
d18:1/22:0	N-(docosanoyl)-sphing-4-enine
d18:1/22:1	N-(13Z-Docosenoyl)-sphing-4-enine-1-phosphocholine
d18:1/23:0	N-(tricosanoyl)-sphing-4-enine
d18:2/23:0	N-(tricosanoyl)-4E,14Z-sphingadienine
d18:1/24:0	N-(tetracosanoyl)-sphing-4-enine
d18:1/24:1	N-(15Z-tetracosenoyl)-sphing-4-enine
d18:1/25:0	N-(pentacosanoyl)-sphing-4-enine
d18:1/26:0	N-(hexacosanoyl)-sphing-4-enine
d18:1/26:1	N-(17Z-hexacosenoyl)-sphing-4-enine

Other lipid nomenclature such as, GPL use a ‘’sn’’ notation which means stereospecific numbering. It means that the hydroxyl group of second carbon of glycerol (sn-2) is on the left side of a Fischer projection (a two-dimensional representation of an organic molecule by projection). The LIPID MAPS GPL abbreviations (PC, PE) are used to refer to species with one or two radyl side-chains where the structures of the side chains are indicated within parentheses in the

'Headgroup' format for example PC (16:0/18:1(9Z)). The 'O'-prefix is used to indicate the presence of an alkyl ether substituent such as PC (O-36:1).

If the GPL side chain regiochemistry and stereochemistry is not known but have information on total composition then abbreviation such a PC (36:1) may be used to show total number of carbon and double bonds for all chains (Fahy et al., 2009).

5.1.6 Lipid Metabolic Pathways

Four metabolic networks have been documented to play an important role in the pathogenesis of disease including SL, GPL, GL and non-esterified fatty acids (NEFA). This thesis focuses more on SL, especially Cer.

5.1.6.1 Sphingolipid Metabolic Pathway

Being one of the important components of a cell membrane, SL play an important role in many cellular functions such as proliferation, maturation, apoptosis and cellular stress response (Mao and Obeid, 2008). Among all SL, Cer is considered one of the most important SL which serves as a precursor for other SL like sphingosine and sphingosine-1-phosphate (S1P). Any change in this metabolic network disturbs the SL homeostasis which affects the cell membrane properties leading to failure of membrane protein functions (Paugh et al., 2008). At normal levels, Cer plays an important role in cell cycle arrest, cell growth, differentiation, necrosis, apoptosis, proliferation and stress response. Cer also regulates enzymes such as protein kinase C, raf-1, kinase suppressor of Ras and cellular protease cathepsin. SIP plays a role in proliferation, cell growth, cell migration, inflammation, angiogenesis and resistance to apoptotic cell death (Borodzicz et al., 2015, Markgraf et al., 2016). Cer can be induced by various stimuli like interleukins, interferons, TNF-α, oxidative stress,

ionising radiation and drugs (Borodzicz et al., 2015). Cer is produced by 3 pathway processes: 1) *de novo* synthesis from saturated fatty acids which takes place in endoplasmic reticulum; 2) by conversion of sphingomyelin into Cer by sphingomyelinase enzyme in the cell membrane (sphingomyelinase pathway); and, 3) salvage pathway in lysosomes, that break down complex SL into sphingosine which is reacylated back to Cer (Gulbins and Li, 2006). Of these, the most important pathway for synthesis of Cer is the *de novo* synthesis in which palmitate and serine combine to form 3-ketosphinganine catalysed by a rate-limiting enzyme, serine palmitoyltransferase (SPT). Sphinganine then forms DhCer by the action of the Cer synthase enzyme followed by Cer formation due to the action of the enzyme dihydroceramide desaturase. Cer can also be produced from degradation of sphingomyelins by the enzyme sphingomyelinase (SMase). Cer is degraded by the enzyme ceramidase (CDase) followed by formation of sphingosine which ultimately forms S1P. The sphingosine can be reacylated to form Cer, known as salvage pathway. Any change in the physiological function of enzymes leads to increased or decreased levels of Cer (Figure 5.4) (Blachnio-Zabielska et al., 2012b). SL is abundant in lipid rafts, which are basically subdomains of a plasma membrane containing a high concentration of cholesterol, so any changes in SL will produce changes in lipid raft composition and function altering cholesterol metabolism and trafficking. This also leads to an alteration in cell-cell communication and adhesion (Rog and Vattulainen, 2014, Hakomori and Igarashi, 1995). It has also been documented that increased levels of Cer leads to lipotoxicity and may induce metabolic disorders (Zhang et al., 2013, Summers, 2006).

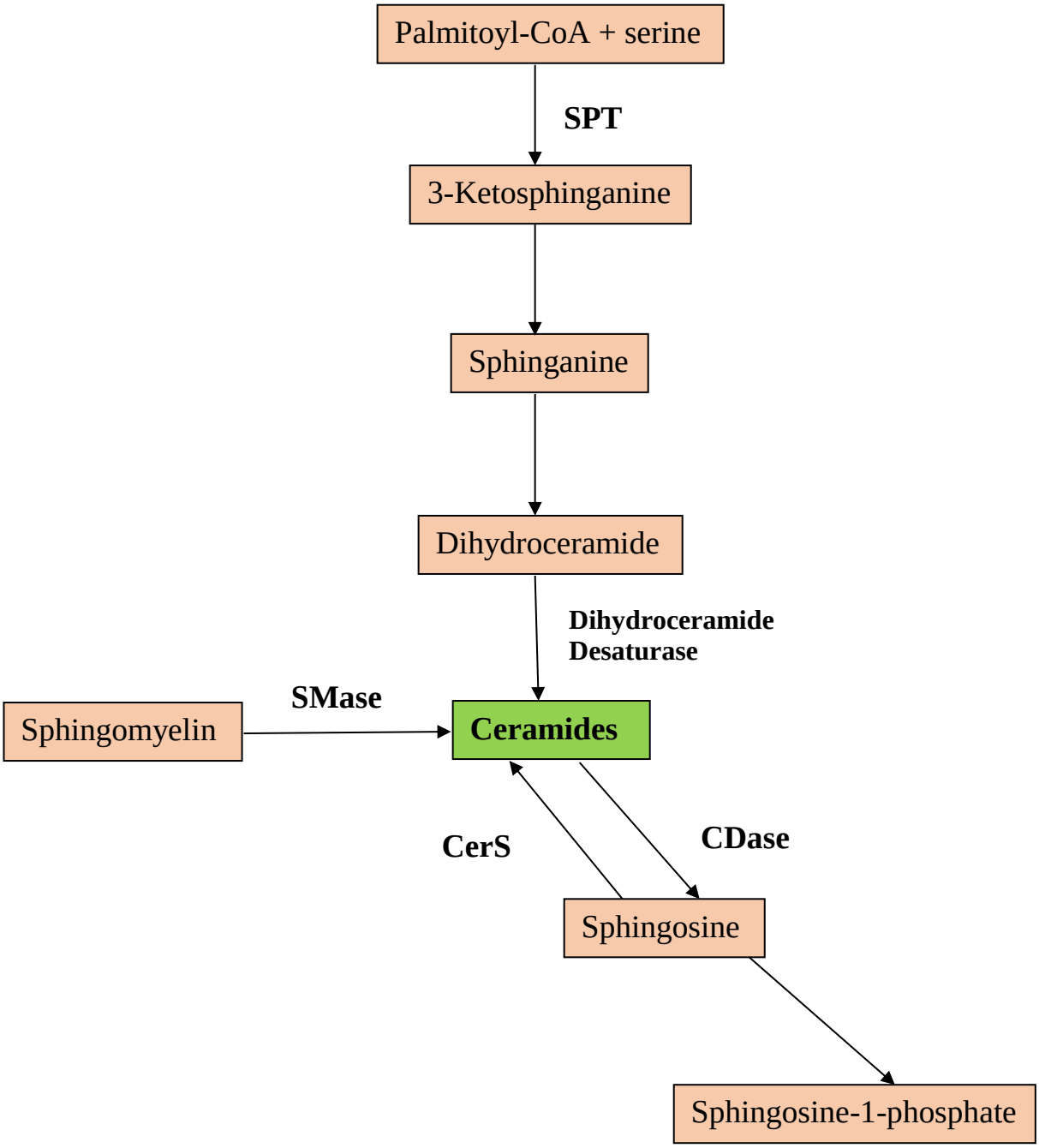


Figure 5.4 Overview of Ceramide metabolism. SPT: serine palmitoyltransferase; SMase: sphingomyelinase; CDase: ceramidase, CerS: ceramide synthase

5.1.7 Role of Sphingolipids in CVD

It has been proposed that factors like infection, inflammation, stress responses, UV light and some chemotherapeutics activate SMase which produces Cer. These factors cause translocation of SMase to an outer layer of the cell membrane which then leads to Cer formation making Cer-enriched membrane domains. These domains cluster receptors on the membrane to amplify signals, which are needed for the induction of apoptosis. The relation between SL, especially Cer, and apoptosis might be important in the pathogenesis of CVD (Schenck et al., 2007). It has been proposed that Cer may cause vasoconstriction and salt and water retention by activation of angiotensin II type 2 receptors resulting in hypertension. An *in vitro* study on rat pheochromocytoma PC12W cells showed activation of angiotensin II type 2 receptors to be accompanied with an increase in Cer and S1P levels (Berry et al., 2001). Spijkers and colleagues' study reported increased levels of Cer in patients with hypertension (Spijkers et al., 2011). It has been suggested that Cer is involved in endothelial dysfunction leading to disturbance in blood flow regulation leading to hypertension (Kulkarni et al., 2017). Cer causes apoptosis of cardiomyocytes and this has been suggested to affect physiological functions of the heart (Borodzicz et al., 2015). Cer is associated with ischemic heart disease; an animal model of myocardial ischemia showed an increased level of Cer compared to the normal control (Zhang et al., 2001). Interestingly, plasma Cer levels and SMase activity are increased in patients with angina pectoris and acute myocardial infarction (Pan et al., 2014). SL roles have also been documented in patients with stroke. A study by Kubota and colleagues showed increased levels of Cer in patients with acute internal carotid artery occlusion (Kubota et al., 1989). It has been suggested that Cer has a tendency for self-aggregation due to its hydrophobicity

and hydrogen bonding capacity. This leads to aggregation of LDL resulting in the formation of atherosclerotic plaques. Cer, due to its proapoptotic mechanism, may lead to the rupture of atherosclerotic plaque causing development of thrombosis leading to ischemia, myocardial infarction, and stroke. Along with Cer, S1P causes thickening of the vascular wall through the proliferation of endothelial and smooth muscle cells which favour plaque stabilisation (Summers, 2006).

5.1.7.1 Role of Sphingolipids in Metabolic Disease

Among all the SL, Cer plays an important role in metabolic disease. Cer, formed from long chain fatty acyl coenzyme A, acts as a toxic lipid on its accumulation in tissues during obesity (Hage Hassan et al., 2014). Cer formation is regulated by Cer synthases (6 types) which, on activation, form a different acyl chain Cer (C14:0-C30:0). Research carried out in Cer synthase-deficient mice showed that altering Cer acyl-chain lengths through deficiency of the specific Cer synthase enzyme may lead to tissue-specific effects (Turpin et al., 2014). In pancreatic beta cells, Cer 18, 22 and 24 formed by Cer synthase type 4, cause apoptosis and blockade of this enzyme was found to halt the apoptotic process. In liver and adipose tissue, Cer 16 and 18 are suggested to be associated with insulin resistance (Turpin et al., 2014, Cinar et al., 2014). In T2DM patients, the expression of Cer 16, 18 and 24 was found to be increased in muscle cells compared to lean individuals (Bajpeyi et al., 2014). Animal and *in vitro* studies have shown an inverse relationship between Cer levels and insulin sensitivity. Cer levels were found to be increased in insulin-resistant models of rodents and mice fed on a high-fat diet, and in intra-lipid infused mice (Samad et al., 2006, Schmitz-Peiffer et al., 1999). The inverse relationship between Cer and insulin sensitivity was also confirmed *in vitro* in cultured C2C12 and L6 myotubes and 3T3-

L1 adipocytes (Hajduch et al., 2001, Schmitz-Peiffer et al., 1999, Summers, 2006). In humans, numerous studies also support the inverse relationship between insulin sensitivity and Cer contents. It has been shown that Cer content was increased and insulin sensitivity decreased in obese, T2DM and cardiovascular disease patients compared to lean individuals (Straczkowski et al., 2004, Amati et al., 2011, Coen et al., 2013, Straczkowski et al., 2007). It has been established that obesity is associated with low-grade inflammation. Cer acts as a link between inflammation and insulin resistance. Saturated fat activates toll-like receptors which increase the transcription of inflammatory cytokines like TNF-α and IL-6 which produce Cer (Hage Hassan et al., 2014). It has been proposed that impairment of mitochondrial function results in the development of toxic lipids like Cer which then lead to insulin resistance (Turpin et al., 2014).

Numerous mechanisms by which Cer causes insulin resistance have been postulated which are discussed below (Fig 5.5).

1) Various studies have shown that Cer decreases insulin-stimulated glucose transport and glycogen synthesis by blocking IRS-1 and PI3K in the insulin signalling pathway (Zundel and Giaccia, 1998, Kanety et al., 1996). However, other studies showed no effect of Cer on these molecules (Hajduch et al., 2001, Summers et al., 1998). Other groups, however, reported PKB/Akt as a target molecule of Cer in the insulin signalling pathway which led to decreased glucose transport. This has been observed in Cer-treated cell types including 3T3-L1 adipocytes, L6 rat and C2C12 mouse skeletal muscle and MCF7 breast cancer cells (Summers et al., 1998, Schmitz-Peiffer et al., 1999, Hajduch et al., 2001).

2) It has been documented that Cer inhibits PKB/Akt by blocking 2 pathways: protein phosphatase 2A (PP2A) and atypical PKC (aPKC). PP2A is a cytoplasmic serine/threonine phosphatase which regulates cellular processes, including metabolic enzymes, kinase cascades, hormone receptors and cell growth. It has been proposed that Cer activates PP2A which causes de-phosphorylation of PKB/Akt, altering insulin signalling pathways (Janssens and Goris, 2001, Salinas et al., 2000). Another pathway by which Cer blocks PKB/Akt is by activation of PKC. PKC makes a complex with PKB/Akt and regulates its physiological action; increased Cer activates PKC which promotes the stabilisation of PKC/PKB/Akt complex and blocks the recruitment of PKB/Akt to the plasma membrane (Powell et al., 2003, Fox et al., 2007).

3) Kinases such as c-Jun N-terminal Kinase (JNK) and I kappa kinase beta (IκKβ) have been reported to regulate insulin signalling. Cer activates JNK and IκKβ resulting in activation of transcription factors Jun and the nuclear factor *kappa*-light-chain-enhancer of activated *B* cells (NF-κB). These factors, in turn, may alter the expression of genes that inhibit proximal signalling events (Summers, 2006, Summers et al., 1998).

4) High Cer concentration increases reactive oxygen species (ROS) which causes mitochondrial dysfunction leading to apoptosis of cells. Mitochondrial dysfunction inhibits lipid oxidation leading to the formation of lipid metabolites that block the insulin signalling pathway (Hage Hassan et al., 2014).

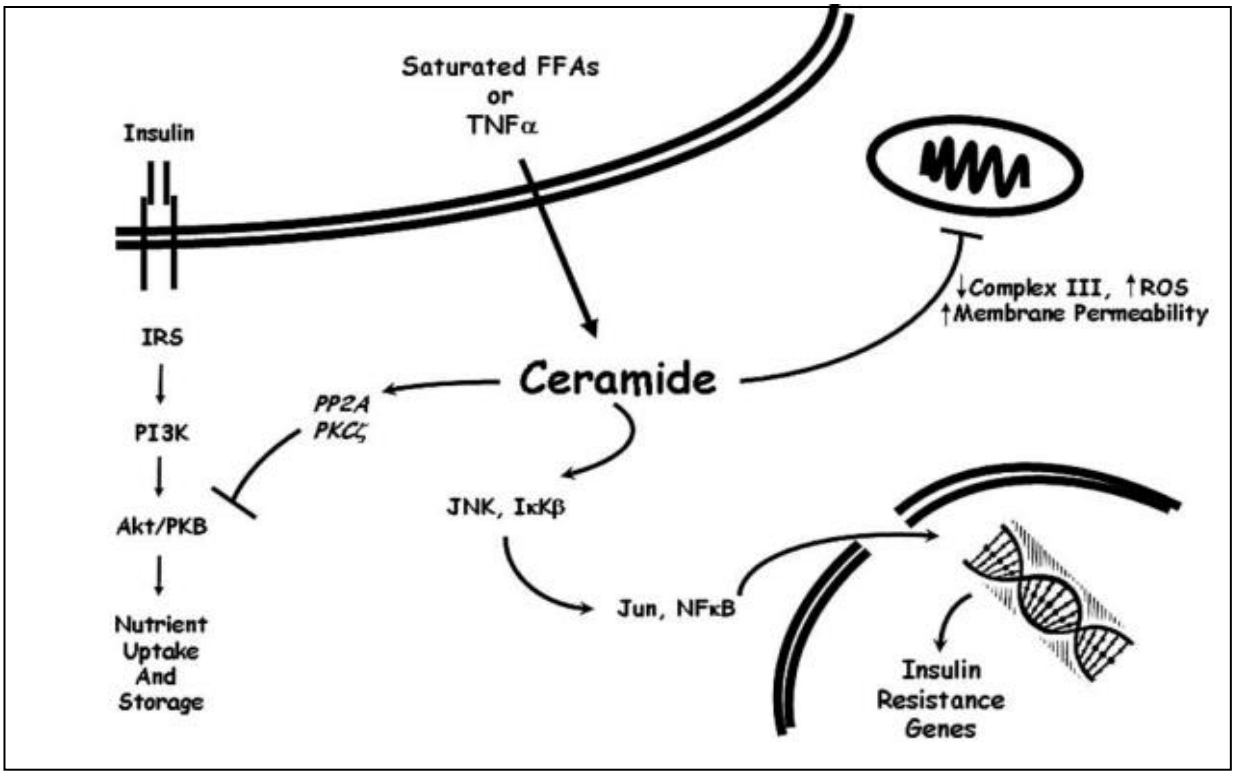


Figure 5.5 Proposed mechanisms by which Cer cause insulin resistance (adapted from Summers SA, 2006).

5.1.8 Lipidomic profiling of Adipocytes

Adipocytes show versatile lipid metabolism machinery which comprises regulation between synthesis, modification, and breakdown. Lipid accumulation in adipocytes starts with fatty acid uptake or lipogenesis from glucose which leads to triglyceride storage in lipid droplets. Lipogenesis is regulated by various enzymes which include acetyl-CoA carboxylase, ATP-citrate lyase, and fatty acid synthase. Lipolysis, in which triglycerides break down to release fatty acids, is regulated by lipoprotein lipase, adipose-triglyceride lipase, and hormone-sensitive lipase. Fatty acids are precursors for all lipid types including PL, SL, sterols, and eicosanoids. Fatty acids are capable of regulating insulin sensitivity in adipocytes. Studies have shown that high levels of PUFA improve insulin sensitivity while high levels of saturated FA reduce insulin sensitivity (Lapid and Graff, 2017). Lipid profiles may change as an

adaptation to various factors like drugs, exercise, and diet or in a pathological condition like T2DM or hepatic steatosis. Therefore, lipids can act as a diagnostic biomarker and can be associated with various diseases like metabolic or cardiovascular disease (Han, 2016). Lipogenesis in adipocytes is regulated by transcriptional factors; most important are PPARγ, C/EBPα, and SREBP1. These factors increase the expression of enzymes that lead to fatty acid biosynthesis, transport and their conversion into TG. These transcriptional factors then activate other factors like fatty acid binding protein (FABP4), liver X receptor (LXR), delta kinase homolog 1 (DLK-1) and kruppel-like factor (KLF), leading to the formation of lipids including various species of FA, prostaglandins, steroid, Cer, and GPL. These transcriptional factors also induce expression of various genes involved in fatty acid and glucose uptake and lipogenesis thus maintaining the normal physiological function of adipocytes (Bergen and Burnett, 2013). Lipogenesis in adipocytes is also controlled by various hormones; insulin is considered one of the main hormones. In adipocytes, insulin promotes the uptake of glucose and its conversion into glycerophosphate which becomes esterified by 3 fatty acids to form triglycerides for storage. In times of caloric requirement, TG converts into FFA which is then transported to the liver and skeletal muscles where it is oxidised to use as an energy source (Hage Hassan et al., 2014) (Fig. 5.6)

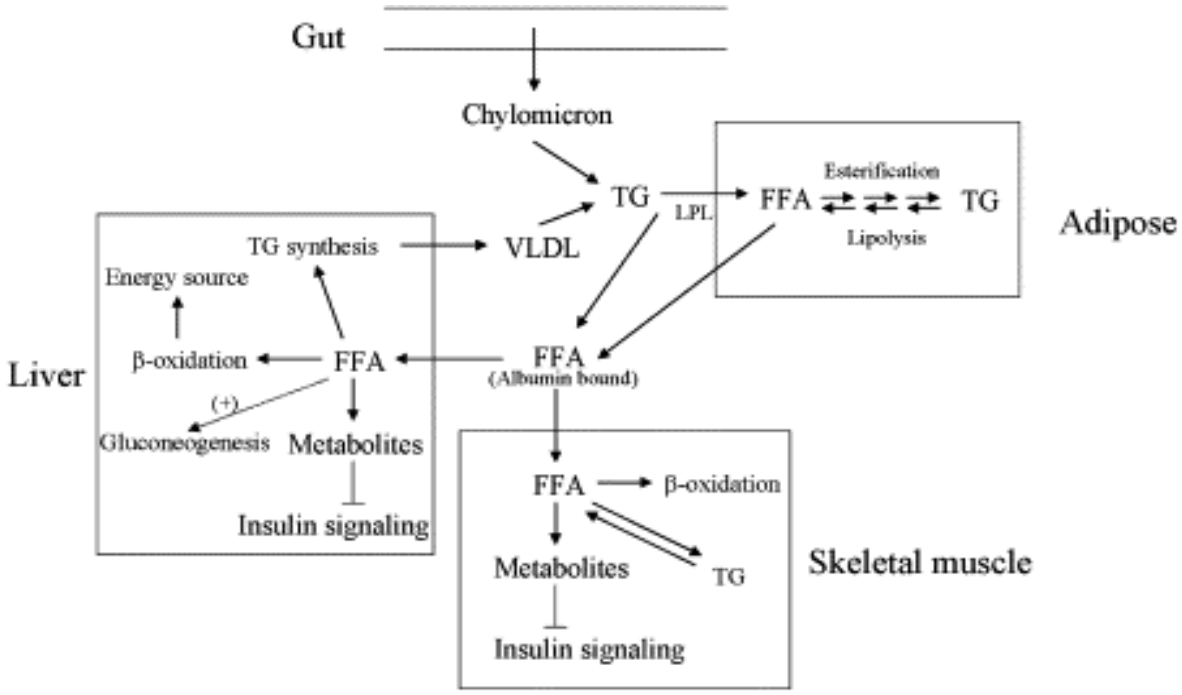


Figure 5.6 Peripheral homeostasis of lipid oxidation for providing energy source (Adapted from Hassan et al.,2014).

As adipocyte is a major organ for lipid metabolism, storage and release, any change in adipocyte lipidome may precede disease development and various lipid markers may be secreted into the blood. Various clinical studies of obesity and diabetes have shown increased levels of DAG, CE, TAG, and Cer in plasma of these patients compared to normal individuals (Tonks et al., 2016, Rauschert et al., 2016). Obesity is one of the main pathological features of metabolic syndrome and is characterised by increased body fat which is stored in adipose tissue. Pietilainen and colleagues proposed the hypothesis, *adipose tissue expandability*, according to which excess fat is stored in adipose tissue but once it reaches its limit, the excess fat is stored ectopically in peripheral tissues such as skeletal muscle, the liver and pancreas (Pietilainen et al., 2011). Lipidomic approaches can be used to characterise various lipid species present in adipose tissue and to understand how changes in these lead to

development of metabolic disease. Various researchers have conducted human studies and shown an association between varied lipid metabolites and obesity and insulin resistance. Pietilainen and colleagues' using obese human adipose tissue, showed an increased association of TG, PC, PE, palmitic and arachidonic fatty acids with obesity. The precursor of arachidonic-derived lipid mediators, such as leukotriens and hydroxyeicosatetraenoic acid, which are important mediators of inflammation, make adipocytes more vulnerable to inflammation (Pietilainen et al., 2011). A study by Jove and colleagues on human subcutaneous adipose tissue showed an association of GPL and SL with obesity. Kolak and colleagues studied the human subcutaneous adipose tissue in obese individuals with a high liver fat content. Out of the 154 lipid species studied, the most prominent appeared to be increased concentration of TAG and Cer, specifically Cer 18, compared to obese individuals with normal liver fat (Kolak et al., 2007). Research has provided information on the role of Cer in insulin resistance not only in skeletal muscle but also in adipocytes. It has been shown that intracellular levels of Cer increase in obese, insulin resistant rats (Turinsky et al., 1990) as well as obese, insulin-resistant humans (Adams et al., 2004). Shah and colleagues' study on mice showed increased levels of Cer in plasma as well as adipose tissue, with an increase of more than 300% for Cer 18 (Shah et al., 2008b). Various studies have shown increased human adipose tissue Cer content in obese, T2DM individuals compared to normal individuals. Among various ceramides, Cer 14, 16, 18 and 24 have been demonstrated to be increased in obese subcutaneous human adipose tissue (Blachnio-Zabielska et al., 2012b, Gertow et al., 2014, Turpin et al., 2014).

5.1.9 Lipid species in Schizophrenia and antipsychotic therapy

There is evidence of lipid abnormalities in many neurological disorders including schizophrenia and bipolar disorder. These abnormalities may cause an alteration in the neurotransmitter receptor function and brain glucose and lipid dysregulation which leads to schizophrenic symptoms. It has been demonstrated that schizophrenia is associated with increased concentration of PC, PE, and FA lipid classes (McEvoy et al., 2013). Schwarz et al have reported a significant increase in the levels of PC and FFA in gray and white matter and a significant increase in Cer in the white matter of schizophrenic patient samples. Interestingly, this study did not show any in FFA and Cer levels after antipsychotic treatment in the white and gray matter of samples compared to untreated schizophrenic controls. In contrast to this, Schwarz et al showed that antipsychotic treatment results in increased Cer concentrations in red blood cell (RBC) samples of schizophrenic patients (Schwarz et al., 2008). Limited research has been done so far to analyse the lipidomic changes in schizophrenic patients taking AAPs. Daouk and colleagues evaluated global plasma lipid changes in schizophrenic patients after 2-3 weeks of treatment with AAPs (OLA, RISP, and ARI). OLA and RISP showed increased concentrations of PC, PE, and TAG and their metabolites compared to ARI (Kaddurah-Daouk et al., 2007). A few studies have also reported the effect of AAPs on n3 and n6 PUFA within PC lipid class. Both n3 (eicosapentaenoic acid) and n6 (arachidonic acid) fatty acids are important for cell-signalling and enzyme regulation. The n6 fatty acids promote inflammatory conditions while n3 fatty acids tend to reduce it. So dysregulation of n3 and n6 fatty acids may divert the cells towards pathological conditions. AAPs, especially CLO and OLA, resulted in increased n6 levels, suggesting inflammation as one of the causes of metabolic toxicity observed in AAP-treated schizophrenic patients (McEvoy et al., 2013, Glen et al., 1996).

5.1.10 Rationale

An in-depth analysis of lipid molecules is crucial for the understanding of cellular physiology and pathology. Together, lipid profiling and computational methods have led to the recognition and involvement of specific lipids in metabolic and cardiovascular diseases (Oresic et al., 2008). Such work may then have the potential to identify lipid biomarkers of disease and /or identify therapeutic targets leading to the development of new drugs (Lapid and Graff, 2017). Adipocytes have been a central interest for the past few years due to their ability to store lipids and endocrine functions along with an established role in the pathogenesis of insulin resistance associated with metabolic diseases such as obesity and T2DM (Hage Hassan et al., 2014). Various studies have shown changes in lipid classes and their metabolites such as PE, PC, CE, TAG and Cer in obese diabetic patients compared to normal individuals (Blachnio-Zabielska et al., 2012b, Kaddurah-Daouk et al., 2007). However, there are only a few studies which have investigated the role of adipocyte lipid species in metabolic toxicity arising as result of AAPs therapy (Kaddurah-Daouk et al., 2007, McEvoy et al., 2013). To our knowledge, there has been no information available on the global effect of AAPs on the lipidome of cultured human subcutaneous adipocytes. Our rationale was to characterise the lipid changes brought about by AAPs in primary human adipocytes and investigate what mechanistic role they play in AAP-induced metabolic toxicity.

5.1.11 Hypothesis

This chapter tests the hypothesis that AAPs cause global changes in the adipocyte lipidome profile and this will contribute to the mechanistic role in AAP-induced metabolic toxicity.

5.1.12 Aims and Objectives

The aims and objectives are:

1) To undertake LC-MS/MS based lipidomic profiling to assess the global changes in adipocyte lipid profile caused by AAPs.

2) To validate selected lipid markers identified from the lipidomic profiling using quantitative gene expression analysis.

5.2 Methods

5.2.1 Materials

The materials were similar as described in section 3.2.1.

5.2.2 Cell Culture

Cell culture was performed as described in section 3.2.2.

5.2.3 Collection of lipid pellets for lipidomic analysis

On day 13, 48 hr after last drug addition, supernatants were aspirated carefully and 2ml of PBS was added to each well twice, shaken briefly and aspirated. 600µl of trypsin was added in each well to detach the cells; this was followed by gentle pipetting to dislodge the cells and the lysate was collected in Eppendorf tubes. These tubes were centrifuged for 1 min at maximum speed to obtain a pellet at the bottom of the tube. The supernatant was discarded and 1ml of PBS was added to the pellet to wash it. The washing process was repeated one more time. After centrifugation for 30 sec, the supernatant was discarded leaving behind pellet only which was frozen at - 80°C for further analysis.

5.2.4 Global lipidomic profiling of cultured primary human adipocytes

Lipidomic profiling was performed at the Lipidomic Research facility, University of Highlands and Islands, Inverness, UK. The methodology used by Folch et al was applied for the extraction of adipocyte lipids (Folch et al., 1957). Briefly, a 50 µl aliquot of cell lysate was extracted with 950µl methanol and 2ml chloroform. 50µL methanol containing internal standards (PC 12:0/12:0, PE 14:0/14:0, Cer 17:0, DAG 17:0/17:0, and TAG 17:0/17:0/17:0) was added and the mixture was then left to stand

at room temperature for 1 hr. 700 µl of 0.1 M KCl was added to partition the samples. The mixture was centrifuged at 2000 x g for 5 min to perform phase separation. The lower chloroform layer was evaporated to dryness under nitrogen gas and reconstituted in 200 µL methanol/chloroform (1/3, v/v) containing 5mM ammonium formate.

5.2.4.1 Liquid chromatography/ Mass spectrometry (LC-MS)

Extracted lipid samples were analysed by LC-MS. All analyses were performed using a Thermo Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempsted, UK) equipped with a heated electrospray ionization probe and coupled to a Thermo Accela 1250 UHPLC system. Samples were injected on to a Thermo Hypersil Gold C18 column (2.1mm x 100 mm, 1.9µm). Mobile phase A consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B consisted of 90:10 isopropanol/acetonitrile containing 10 mM ammonium formate and 0.1% (v/v) formic acid. The flow rate was 400 µl/min. The samples were analysed in positive (2 µL injection) and negative (5 µL injection) ion modes over the mass to charge ratio (m/z) range 250-2000 at a resolution of 100,000 and 1Hz scan speed.

5.2.5 Data Processing and Multivariate data Analysis (MVDA)

The raw LC-MS data were processed with Progenesis QI software (version 2.1, Non-linear Dynamics, Newcastle, UK). Lipid identifications were made using LIPID MAPS (www.lipidmaps.org) and human metabolomics database (HMDB) (http://www.hmdb.ca). MVDA was performed with SIMCA-P v13.0.2 (Umetrics, Umea, Sweden).

In order to analyse systematically and extract important information, MVDA is conducted by which the association between lipid species and its effect on lipid metabolism can be studied. MVDA can be classified into;

1) Unsupervised data analysis known as Principal component analysis (PCA) and,

2) Supervised methods called as Partial least squares discriminant analysis (PLS-DA).

In PCA, the data is projected to observe how the samples are clustering by themselves. Here, prior knowledge or classification of sample groups are not applicable and therefore is a unbiased analysis. PCA can also be used to decrease dimensionality of the data set and show outliers, sample grouping, trends of variation and systematic pattern.

PLS-DA is a supervised method where the information about each sample group is supplied. PLS-DA was used to better discriminate control and treated cells. In other words it can be employed when sample group classification is already known. PLS-DA can be used to find information on major trends, to find out lipid species responsible for the trends and variation among different sample group (Oresic, 2010).

5.2.6 Gene expression

Isolation of mRNA, reverse transcription and gene expression were measured by methodology as described in section 2.2.6.

5.3 Statistical Analysis

All statistical comparisons (5 donors) were made using t-test on StatsDirect software version 2.7.9. Differences were considered significant at $p \leq 0.05$. The result was shown as mean ± SD.

5.4 Results

5.4.1 Global lipidomic analysis by MVDA

5.4.1.1 Effect of CLO on global lipidomic analysis

For CLO, both the PCA and the PLS-DA showed a clear separation between the three groups (Group 1- Vehicle; *yellow*; Group 2 – 1µM CLO, *purple*; Group 3 – 20µM CLO, *brown*) analysed (Fig 5.7 A-B). The separation of clusters were more evident in the samples which were analysed under negative ion mode (Fig 5.7 A-B). Even though there was some separation between the Vehicle and 1µM CLO clusters, these clusters were closely located suggesting the global differences in lipid profiles between vehicle (control) and those treated with therapeutic doses of CLO were not significant. However, samples treated with 20µM CLO cluster (Group 3, *brown*) showed a clear separation from both vehicle and 1µM CLO and were in a different quadrant. We also saw some degree of variability between the 5 donor adipocyte used depending on the ion modes used; whilst donors 0092, 511 and 101 displayed similar characteristics and clustered in the same quadrant, donors 400 and 395 were outliers (Fig 5.7 A on positive ion mode). Donor 101 was also repeated (shown as 101R in the Fig 5.7 A-B) and was used as a technical replicate. In the negative ion mode PCA, the samples showed lesser variability; this may suggest the variability was not only sample specific but also lipid related. PLS-DA analysis showed a better separation between groups analysed (Fig 5.7 B).

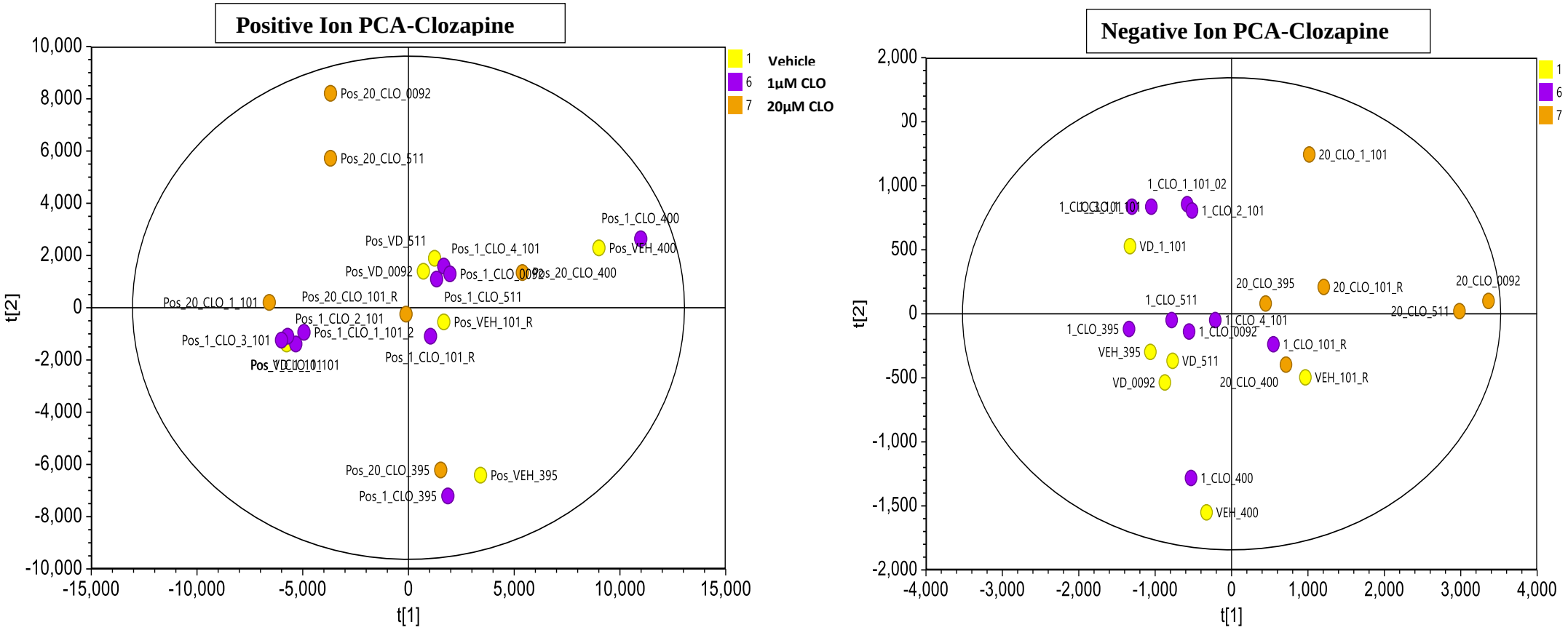


Figure 5.7: A) PCA analysis of cellular lipids in CLO treated samples of cultured primary human adipocytes in both positive and negative ion mode. PCA: Principal component analysis, Pos: Positive ion mode, VEH/VD: Vehicle. Circles denoted as; Yellow: *Vehicle*, Purple: *1µM CLO*, Brown: *20µM CLO*.

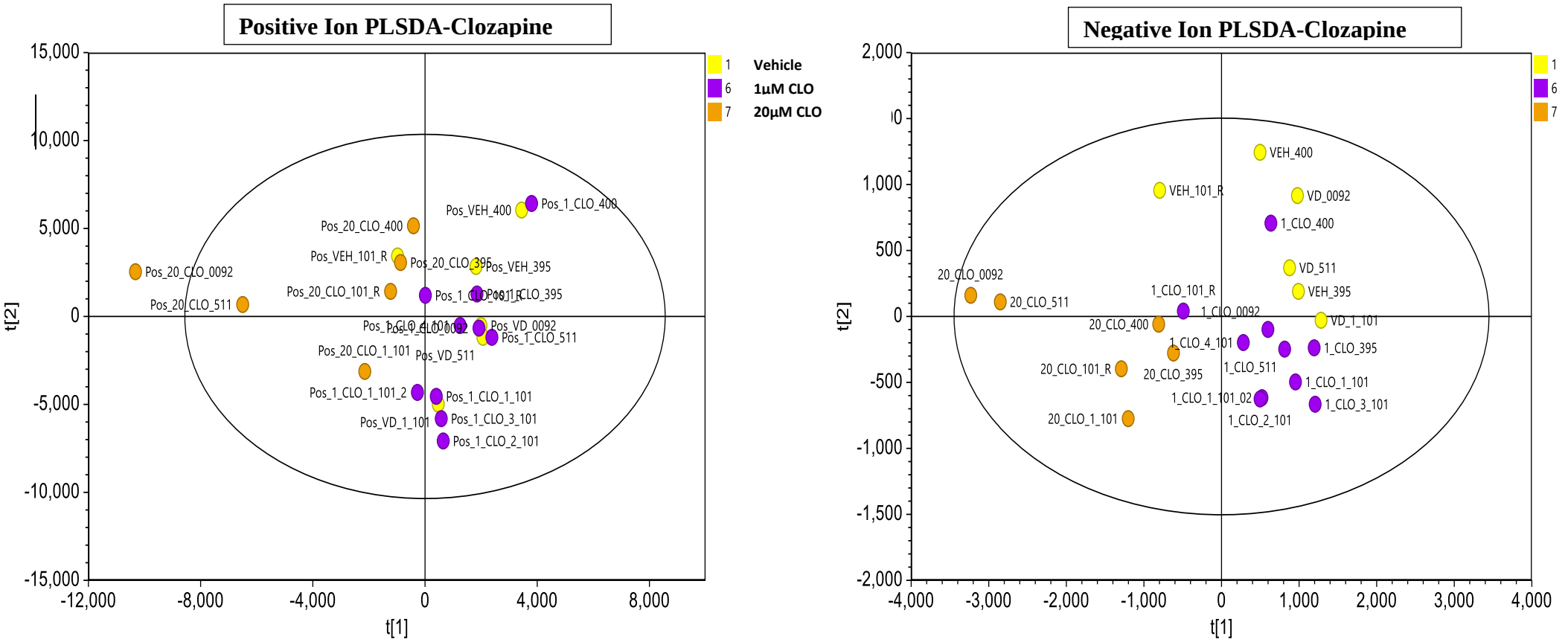


Figure 5.7: B) PLS-DA analysis of cellular lipids in CLO treated samples of cultured primary human adipocytes in both positive and negative ion mode. PLS-DA: Partial least squares discriminant analysis, Pos: Positive ion mode, VEH/VD: Vehicle. Circles denoted as; Yellow: *Vehicle*, Purple: *1µM CLO*, Brown: *20µM CLO*.

5.4.1.2 Effect of OLA on global lipidomic analysis

For OLA, both the PCA and the PLS-DA showed a clear separation between the three groups (Group 1- Vehicle; *yellow*; Group 2 – 0.2µM OLA, *dark blue*; Group 3 – 20µM OLA, *light blue*) analysed (Fig 5.8 A-B). The separation of clusters were more evident in the samples which were analysed under negative ion mode (Fig 5.8 A-B). Even though there was some separation between the Vehicle and 0.2µM OLA clusters, these clusters were closely located suggesting the global differences in lipid profiles between vehicle (control) and those treated with therapeutic doses of OLA were not significant. However, samples treated with 20µM OLA cluster (Group 3, *light blue*) showed a clear separation from both vehicle and 0.2µM OLA and were in a different quadrant. We also saw some degree of variability between the 5 donor adipocyte used depending on the ion modes used; whilst donors 0092, 395, 511 and 101 displayed similar characteristics and clustered in the same quadrant, donor 400 was outlier (Fig 5.8 A on positive ion mode). Donor 101 was also repeated (shown as 101R in the Fig 5.8 A-B) and was used as a technical replicate. In the negative ion mode PCA, the samples showed lesser variability; this may suggest the variability was not only sample specific but also lipid related. PLS-DA analysis showed a better separation between groups analysed (Fig 5.8 B).

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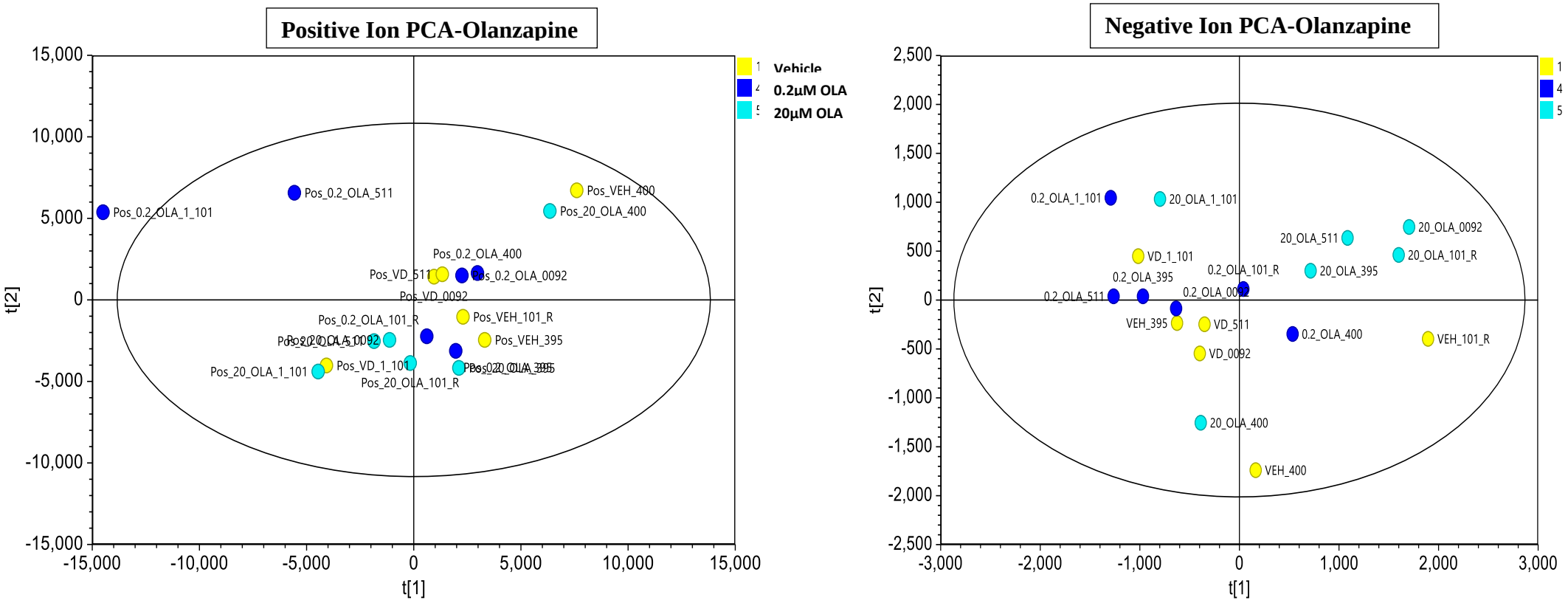

Figure 5.8: A) PCA analysis of cellular lipids in OLA treated samples of cultured primary human adipocytes in both positive and negative ion mode. PCA: Principal component analysis, Pos: Positive ion mode, VEH/VD: Vehicle. Circles denoted as; Yellow: *Vehicle*, Dark blue: *0.2µM OLA*, Light blue: *20µM OLA*.

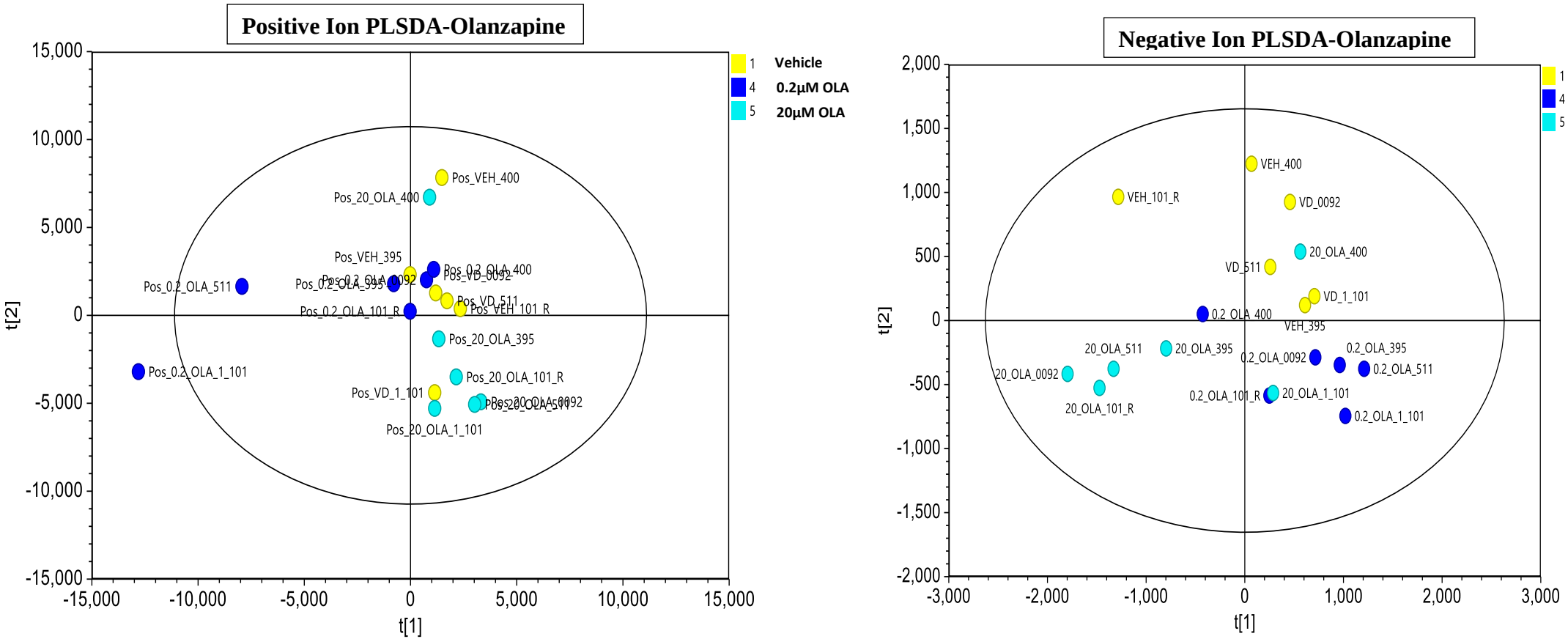

Figure 5.8: B) PLS-DA analysis of cellular lipids in OLA treated samples of cultured primary human adipocytes in both positive and negative ion mode. PLS-DA: Partial least squares discriminant analysis, Pos: Positive ion mode, VEH/VD: Vehicle. Circles denoted as; Yellow: *Vehicle*, Dark blue: *0.2µM OLA*, Light blue: *20µM OLA*.

5.4.1.3 Effect of ARI on global lipidomic analysis

For ARI, both the PCA and the PLS-DA showed lesser separation between the clusters of three groups (Group 1- Vehicle; *yellow*; Group 2 – 0.2µM ARI, *green*; Group 3 – 10µM ARI, *red*) analysed (Fig 5.9 A-B). This was more evident in the samples which were analysed under negative ion mode (Fig 5.9 A-B). Even though there was some separation between the Vehicle, 0.2µM ARI and 10µM ARI clusters, these clusters were closely located suggesting the global differences in lipid profiles between vehicle (control) and those treated with therapeutic and the high dose of ARI were not significant. However there was clear difference between vehicle and high dose of ARI of donor 0092 and 511 showing global differences between them but should be viewed with caution as it was only seen with one sample (Fig 5.9A-B). We also saw some degree of variability between the 5 donor adipocyte used depending on the ion modes used; whilst donors 0092, 511, 101 and 400 displayed similar characteristics and clustered in the same quadrant, donors 395 was outlier (Fig 5.9 A on positive ion mode). Donor 101 was also repeated (shown as 101R in the Fig 5.9 A-B) and was used as a technical replicate. In the negative ion mode PCA, the samples showed lesser variability; this may suggest the variability was not only sample specific but also lipid related. PLS-DA analysis showed a better separation between groups analysed (Fig 5.9 B).

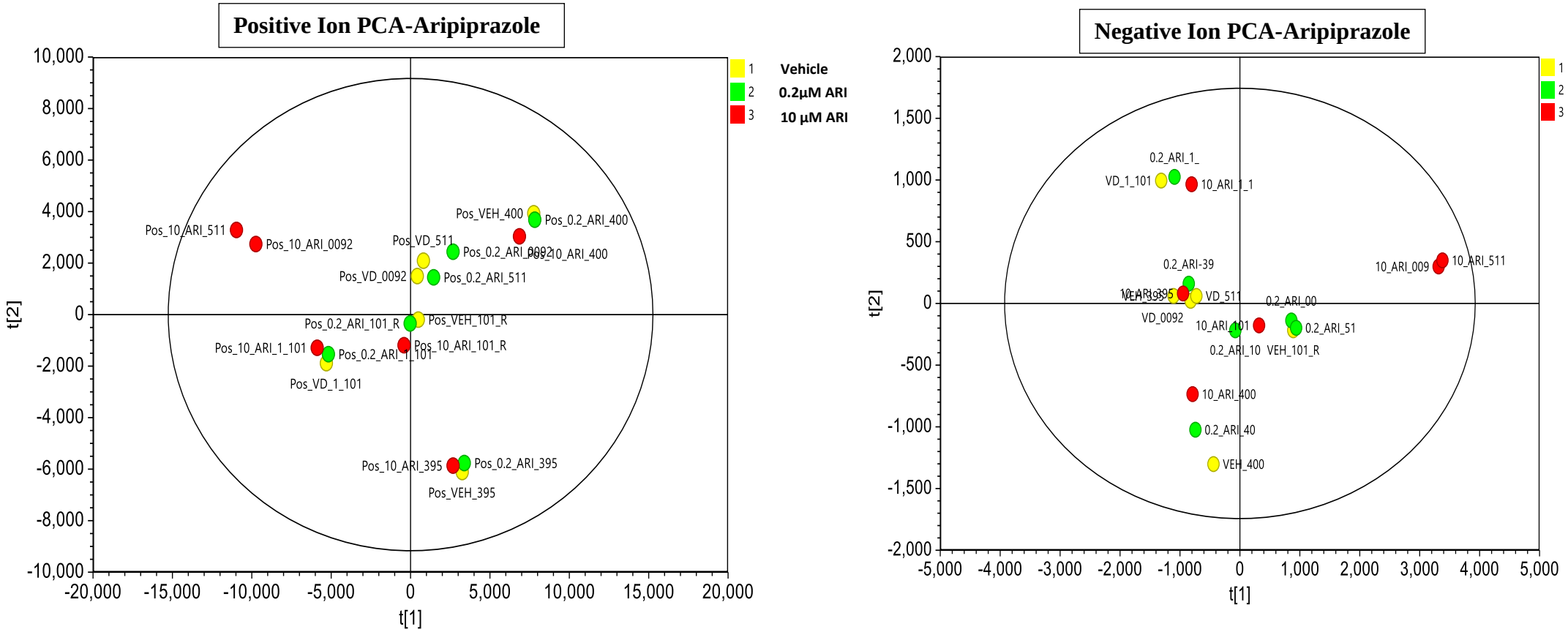

Figure 5.9: A) PCA analysis of cellular lipids in ARI treated samples of cultured primary human adipocytes in both positive and negative ion mode. PCA: Principal component analysis, Pos: Positive ion mode, VEH/VD: Vehicle. Circles denoted as; Yellow: *Vehicle*, Green: *0.2µM ARI*, Red: *10µM ARI*.

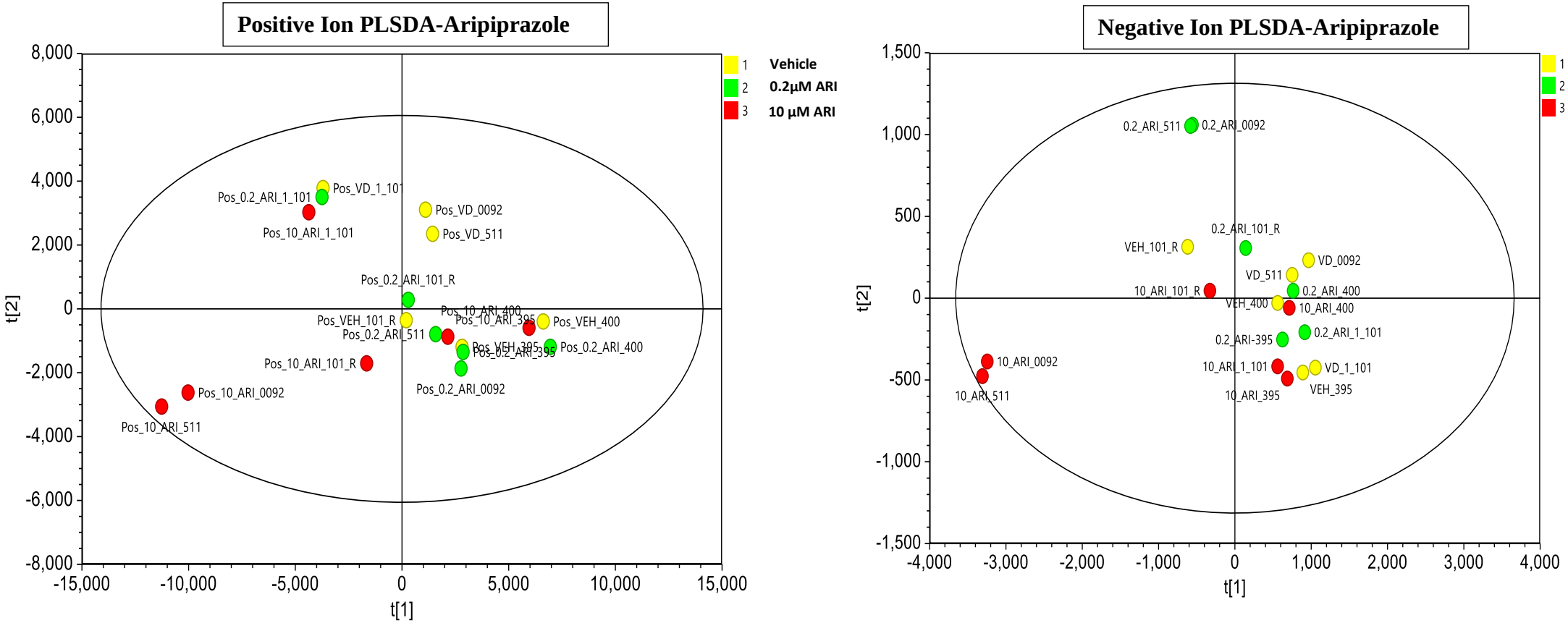

Figure 5.9: B) PLS-DA analysis of cellular lipids in ARI treated samples of cultured primary human adipocytes in both positive and negative ion mode. PLS-DA: Partial least squares discriminant analysis, Pos: Positive ion mode, VEH/VD: Vehicle. Circles denoted as; Yellow: *Vehicle*, Green: *0.2µM ARI*, Red: *10µM ARI*.

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Of the various lipid species detected, we focussed on the Cer class of species to investigate the effect of AAPs.

5.4.2.1 Effect of CLO on Cer lipid species

Our data showed that the therapeutic dose of CLO (1µM) induced a significant decrease in all of major Cer species except Cer 17:0 and Cer 19:0 in comparison with the vehicle. The higher dose of CLO (20µM) also resulted in significant decrease in various Cer species listed in brackets (16:1, 18:0, 20:0, 22:0, 22:1, 23:0, 24:0, 24;1, 26:0 and 26:1). Cer 16:0 and 25:0 also showed decreased levels but the result was statistically non-significant. One of the Cer species (18:1) could not be detected in all the samples while 17:0 and 19:0 species showed no change after giving high dose of CLO (Fig 5.10A-D).

Comparison between 1µM and 20µM CLO showed a trend to increase for various Cer species (16:0, 17:0, 18:0, 20:0, 22:0, 23:0, 24:0, 25:0, 26:0 and 26:1); however, this was non-significant. Cer 16:1, 19:0, 22:1 and 24:1 did not show any change on comparison between therapeutic and high dose of CLO. Cer 18:1 was not be detected in all of the donors following treatment with high dose of CLO (20µM) and was only detected in adipocytes treated with the therapeutic dose of CLO (1µM) (Fig 5.10 A-D).

5.4.2.2 Effect of OLA on Cer lipid species

Both therapeutic and high dose of OLA showed significant decrease in all of the Cer species except 17:0 and 19:0 which showed no change compared to vehicle (Figure 5.10 A-D).

Comparison between 0.2µM and 20µM OLA showed a trend to increase for various Cer species (16:0, 18:0, 20:0, 22:0, 22:1, 23:0, 24:1, 25:0, 26:0 and 26:1) but the result was non-significant. However Cer 16:1, 17:0, 18:1, 19:0 and 24:0 did not show any change on comparison between therapeutic (0.2µM) and high dose (20µM) of OLA (Fig 5.10 A-D).

5.4.2.3 Effect of ARI on Cer lipid species

In contrast to the Cer results obtained with CLO and OLA, therapeutic dose of ARI (0.2µM) showed a trend to increase all of the Cer species levels; however, this was statistically non-significant. Higher dose of ARI (10µM) did not show any effect in any of the Cer species except Cer 18:1 and Cer 20:0; both these Cer showed a significant decrease in comparison to the vehicle.

Comparison between therapeutic and high dose of ARI showed a trend to decrease in all of the measured Cer species by high dose of ARI except Cer 17:0, there was no change; however the result was non-significant (Figure 5.10 A-D).

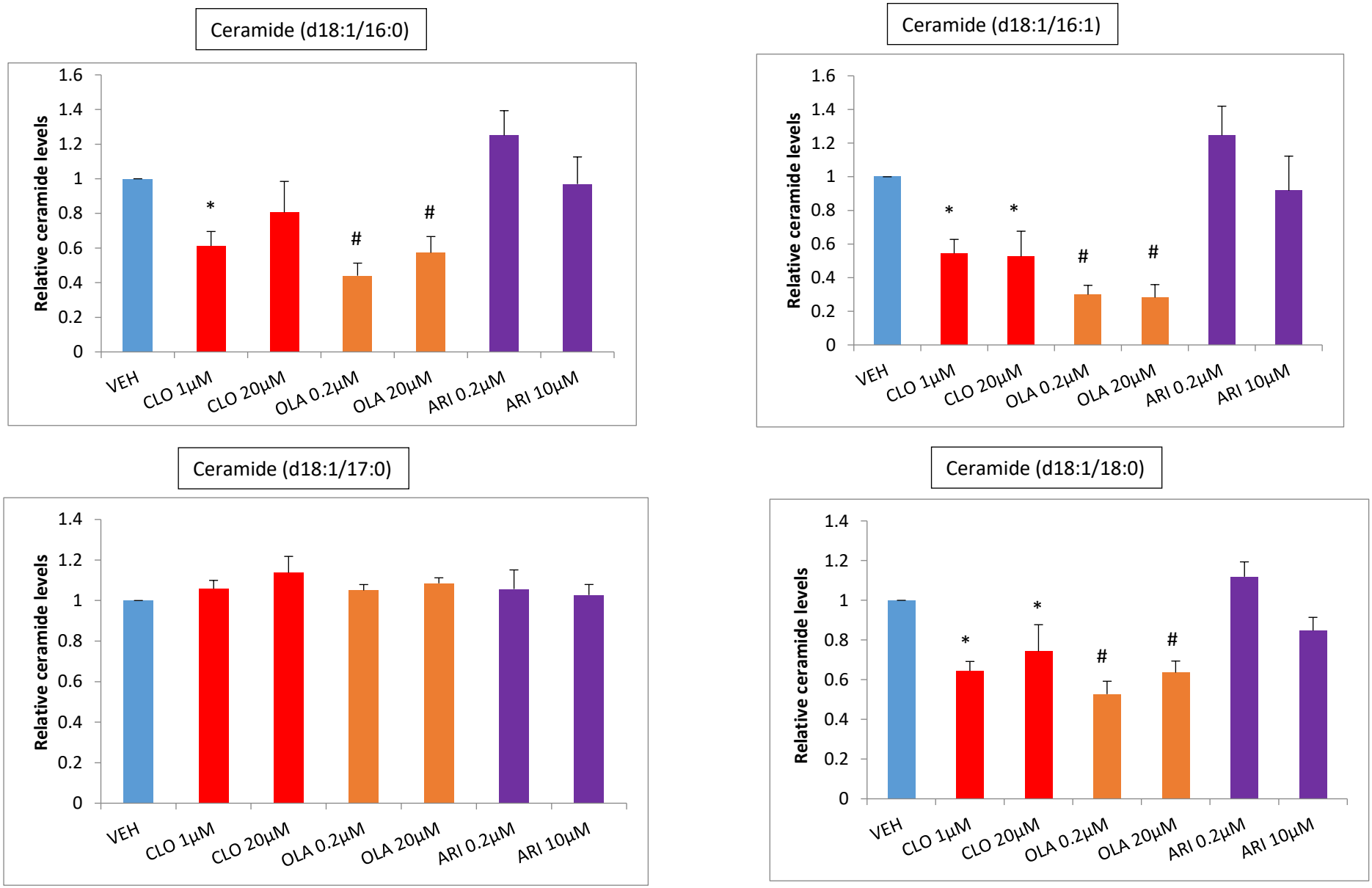

Figure 5.10: A) Ceramide (d18) subspecies (16:0, 16:1, 17:0, 18:0) levels showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 5 individual donors. Data was shown as mean ± SD; $p \leq 0.05$. * VEH vs CLO, # VEH vs OLA.

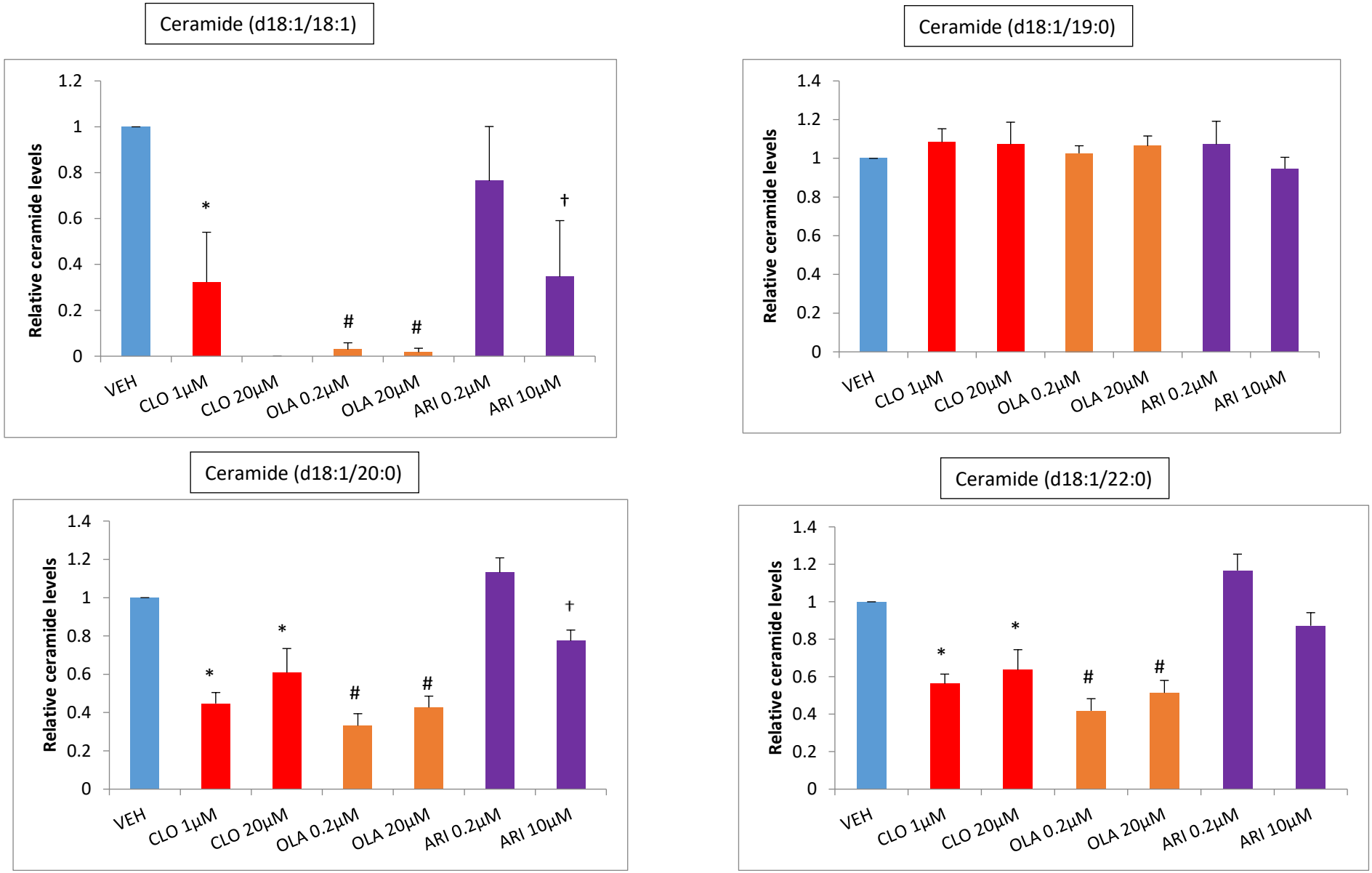

Figure 5.10: B) Ceramide (d18) subspecies (18:1, 19:0, 20:0, 22:0) levels showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 5 individual donors. Data was shown as mean ± SD; $p \leq 0.05$. * VEH vs CLO, # VEH vs OLA, † VEH vs ARI.

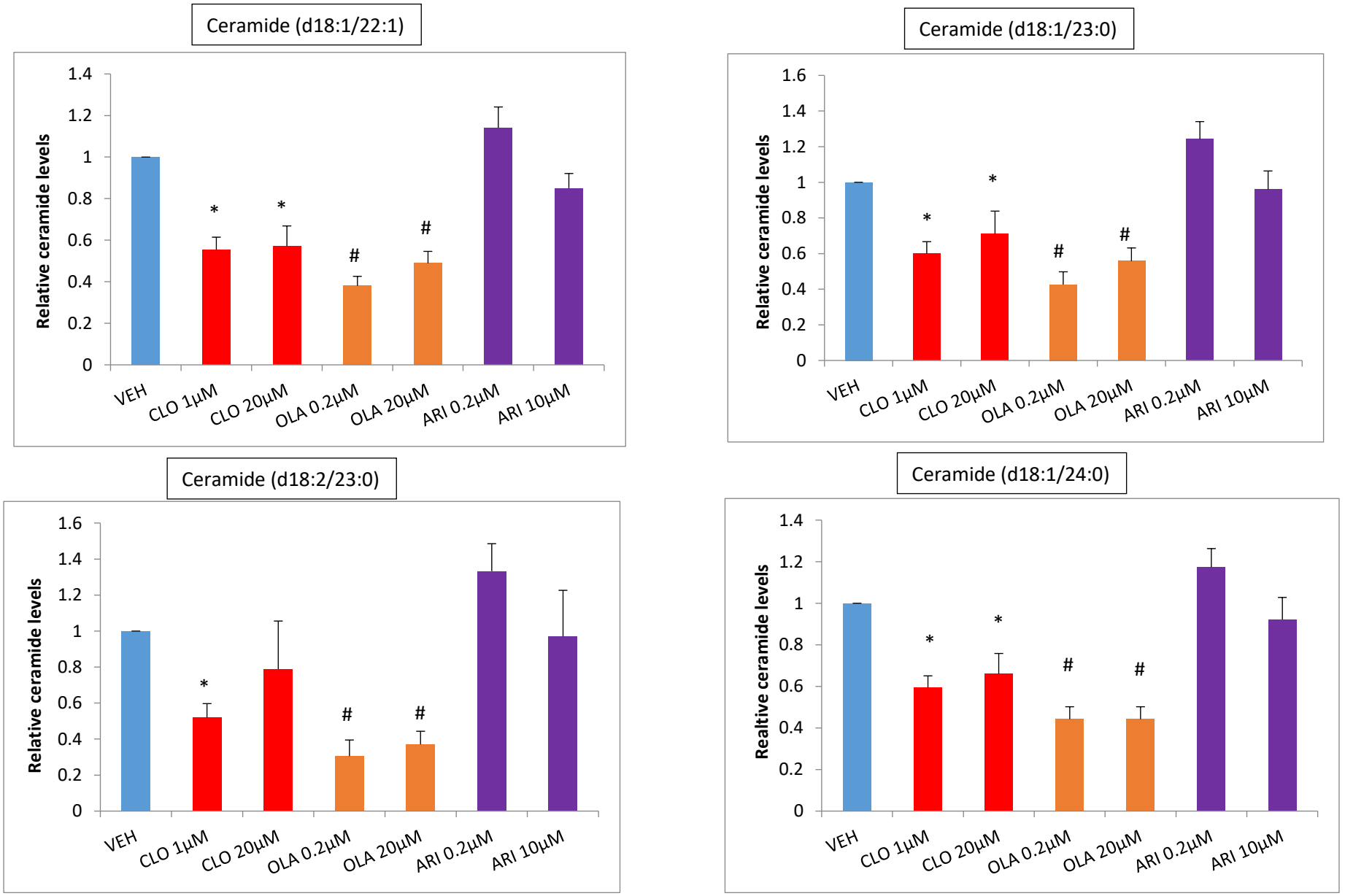

Figure 5.10: C) Ceramide (d18) subspecies (22:1, 23:0, d18:2/23:0, 24:0) levels showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 5 individual donors. Data was shown as mean ± SD; $p \leq 0.05$. * VEH vs CLO, # VEH vs OLA.

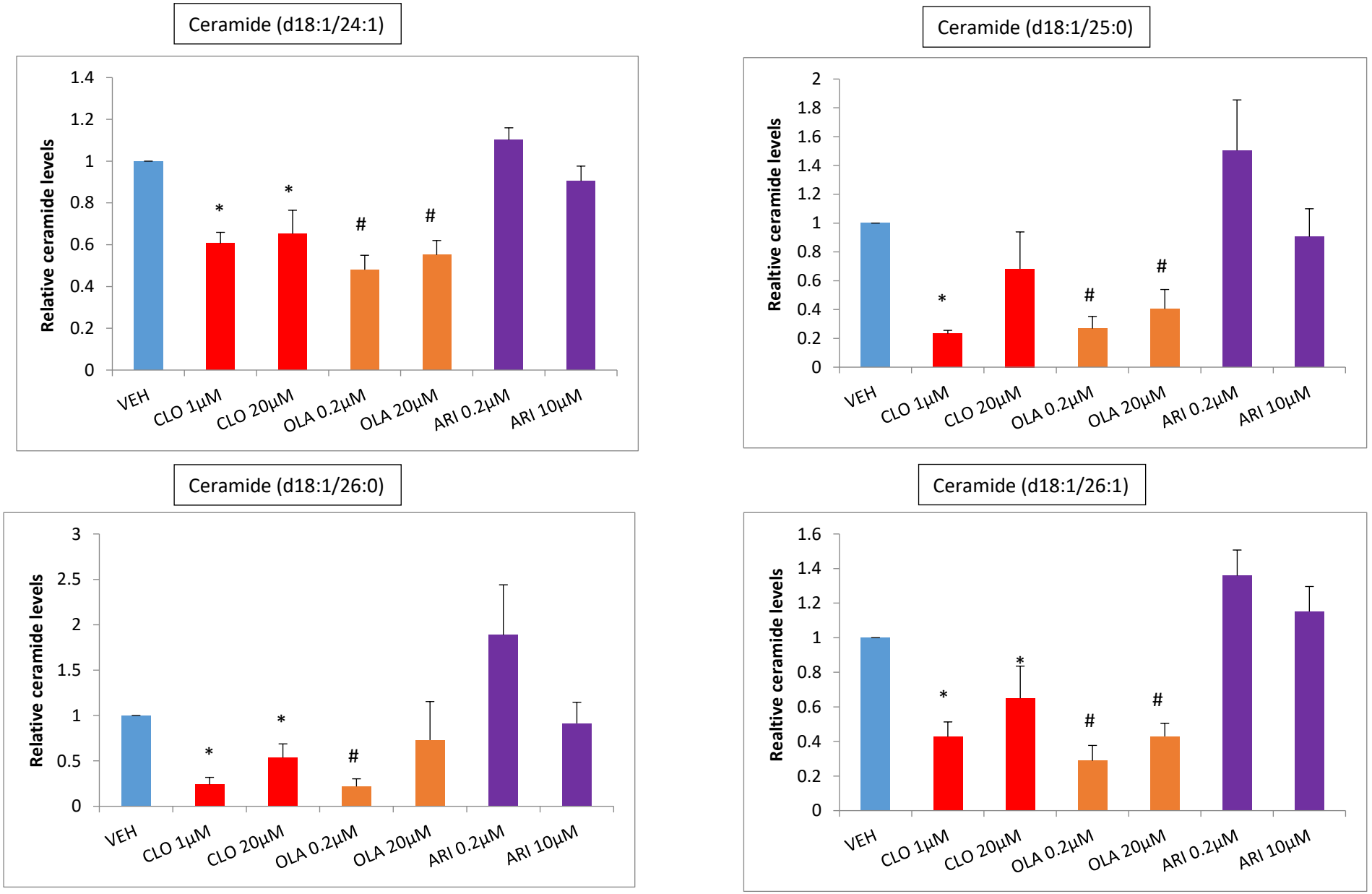

Figure 5.10: D) Ceramide (d18) subspecies (24:1, 25:0, 26:0, 26:1) levels showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 5 individual donors. Data was shown as mean ± SD; $p \leq 0.05$. * VEH vs CLO, # VEH vs OLA.

5.4.3 Validation of Cer data

To validate the Cer species data, we investigated the Cer biosynthesis pathway and selected 2 enzymes, Serine Palmitoyltransferase Long Chain Base Subunit 2 (SPTLC2) and Dihydroceramide desaturase (DEGS1) as they both are involved in Cer synthesis. SPTLC2 is considered as a rate limiting enzyme which play role in *de novo* synthesis of Cer. We investigated the effect of AAPs on SPTLC2 and DEGS1 gene expression as an indirect method for validation of results obtained for Cer lipid species.

5.4.3.1 Effect of AAPs on *SPTLC2* and *DEGS1* gene expression

SPTLC2 and *DEGS1* mRNA expression for each drug and dose is presented as a percentage of what was observed in comparison with the vehicle. The therapeutic dose of CLO (1µM) showed a trend to decrease *SPTLC2* and *DEGS1* expression although this was not statistically significant (Mean fold change±SD; *SPTLC2*:0.84±0.16; p=0.06, *DEGS1*:0.75±0.24; p=0.07). However, the higher dose of CLO (20µM) showed a significant decrease in the expression of both enzymes (*SPTLC2*: 0.60±0.13; p=0.003; *DEGS1*: 0.67±0.18; p=0.01). OLA on the other hand, showed significant decrease in expression of both enzymes by therapeutic as well as high dose (*SPTLC2* [0.2µM: 0.77±0.15; p=0.05, 20µM:0.62±0.16; p=0.007], *DEGS1* [0.2µM:0.67±0.25; p=0.04, 20µM:0.83±0.11; p=0.02]). On the contrary, therapeutic dose of ARI(0.2µM) showed a trend to increase both *SPTLC2* and *DEGS1* expression although this was not statistically significant (*SPTLC2*:1.11±0.20;p=0.06, *DEGS1*:1.27±0.21;p=0.06).

However, high dose of ARI (10μM) showed an opposite effect to the therapeutic dose; it resulted in a significant decrease in *DEGS1* expression (0.68±0.19; p=0.05) and showed a trend to decrease *SPTLC2* expression (0.60±0.30; p=0.1) (Fig.5.11 A-B).

Fig. 5.11 A

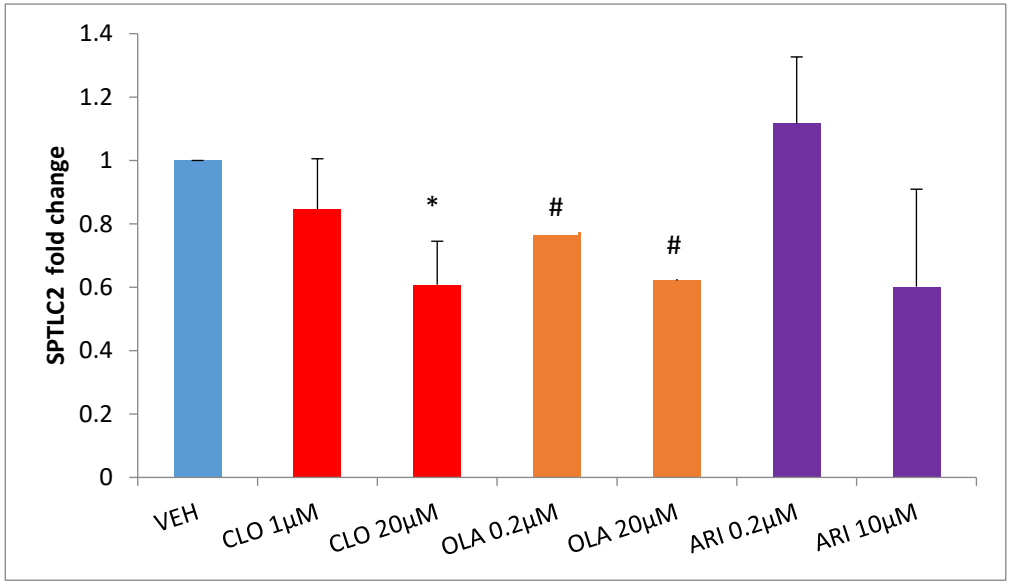

Fig. 5.11 B

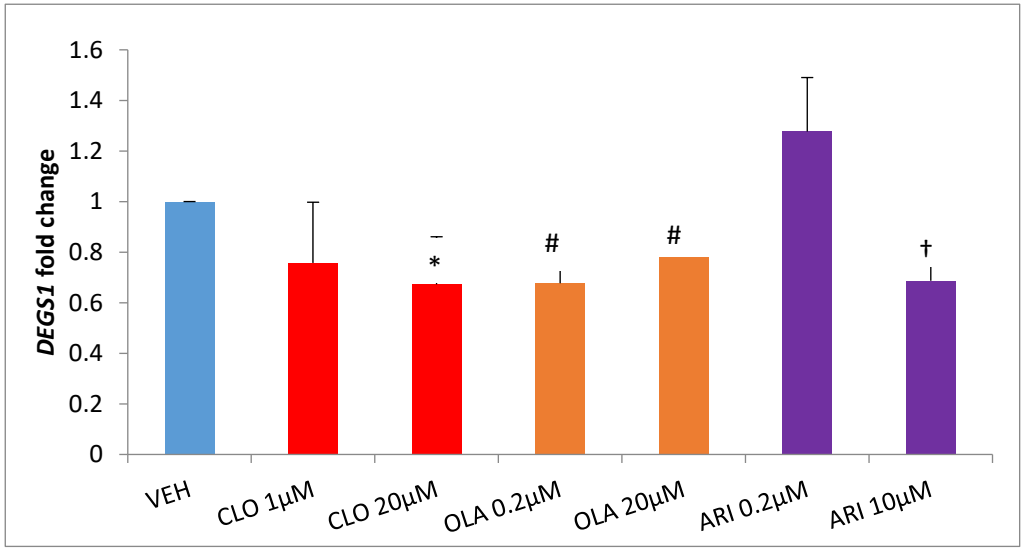

Figure 5.11: A) *SPTLC2* and B) *DEGS1* gene expression showing the effect of various concentrations of CLO, OLA and ARI on differentiated adipocytes. All experiments were done in adipocytes cultured from 5 individual donors. Data was shown as mean ±SD; p≤0.05. * VEH vs CLO, # VEH vs OLA, † VEH vs ARI.

5.5 Discussion

The study of lipids at the global level in adipocytes is a new but rapidly growing field which has the potential to uncover potential pathways and newer biomarkers which may be of use in clinical practice. The composition of lipids in adipocytes and their levels change with external perturbations may show of the various metabolic pathways which are of significance to normal glucose and lipid homeostasis as well as cardiometabolic disease pathogenesis. Understanding of global changes in lipids in complex diseases such as schizophrenia and the potential changes in their levels during treatment with AAPs could provide information about the mechanism of disease and response to drugs. (Kaddurah-Daouk et al., 2007). SL, considered as one of the key classes of lipids, are active components of cell membranes. SL regulates cellular processes such as proliferation, maturation, and apoptosis. Among SL, Cer is considered as one of the most important lipid species which regulates cellular functions ranging from proliferation, differentiation, growth arrest and apoptosis (Hage Hassan et al., 2014). Various studies have shown an inverse association of Cer with insulin sensitivity in metabolic and cardiovascular diseases (Turpin et al., 2014, Blachnio-Zabielska et al., 2012b). AAPs have been documented to cause various metabolic adverse effects in schizophrenic patients. Among various mechanisms, insulin resistance is considered one of the main mechanisms by which AAPs cause metabolic adverse effects such as obesity, T2DM and CVD (Bartoli et al., 2015b, Liu et al., 2014). The data reported here represent the first attempt to map global lipid changes in *in vitro* primary human adipocytes following treatment with CLO, OLA, and ARI. Our multivariate data analysis showed that AAPs resulted in changes in lipid levels in cultured primary human adipocytes. We found the therapeutically relevant concentrations of CLO and OLA caused little change in the global lipid profile in a

differentiating primary human adipocyte. However, the higher dose of CLO and OLA had a more profound effect on the lipidome of the adipocyte. On the other hand therapeutic dose of ARI did not show much a change in the global lipid profile. This suggests that CLO and OLA, which are notorious for causing metabolic adverse effects cause global changes in lipids when at higher concentrations particularly, and these may play a role in causing metabolic toxicity. This supports our hypothesis that some AAPs such as CLO and OLA when administered chronically could accumulate in the adipose tissue and cause deleterious effects which may explain the mechanisms behind AAP-induced cardiometabolic toxicity. This also coincided with Daouk et al's study which showed that OLA brought about changes (increase) in lipids; Daouk's study showed approximately 50 lipids were increased, while ARI showed fewer changes. Although Daouk et al investigated plasma samples obtained from schizophrenia patients whereas we interrogated *in vitro* samples, both studies suggest that AAPs associated with metabolic risk caused more disturbances in the lipid profile than those with reduced metabolic toxicity.

We then further focussed on an individual lipid class, the ceramides. Previous studies have shown changes in lipid classes and subclasses in metabolic deranged conditions like obesity and T2DM; however, it has been suggested that these patients showed more changes in GLP and SL levels compared to other lipids (Alshehry et al., 2016). Among SL, Cer showed an association with CVD (Stegemann et al., 2014, Spijkers et al., 2011). Cer are also documented as being associated with obesity and insulin resistance where its levels are increased (Nestel et al., 2014, Tonks et al., 2016). There is an inverse relationship between Cer and insulin sensitivity. This has been confirmed in various *in vitro* studies using 3T3-L1 adipocytes, L6 myotubes and C2C12 cultured

cells (Hajduch et al., 2001, Schmitz-Peiffer et al., 1999, Summers, 2006). A rodent model of insulin resistance (Samad et al., 2006) and clinical studies on insulin resistant obese and T2DM patients (Coen et al., 2010, Amati et al., 2011) have also shown increased levels of Cer compared to normal. This inclined us to look at the effect of AAPs on the Cer profile in our primary human adipocytes model. There are many Cer species; however, it has been suggested that Cer 16 and 18 are associated with insulin resistance (Turpin et al., 2014). Various preclinical (Shah et al., 2008b) and human obese and high fat studies (Kolak et al., 2007, Blachnio-Zabielska et al., 2012b) showed Cer 18 species to be increased in adipose tissue which inclined us to focus on this Cer species. Shah et al preclinical study in high fat diet mice showed a 300% increase in Cer 18 in adipose tissue which showed its association with metabolic disease (Shah et al., 2008a). Our data showed a decrease in Cer species (16:0, 18:0, 18:1, 20:0, 22:0, 22:1, 24:0 ,24:1, 26:0, 26:1) on giving CLO and OLA while ARI showed no change compared to the control.

Surprisingly, our data did not coincide with other studies which showed increased Cer levels in obese, T2DM patients. Tonks and colleagues' study showed increased plasma and skeletal muscle Cer levels in obese insulin-resistant individuals. Although their study showed changes in all Cer 18 subclasses, the most prominent were related to 18:0, 24:0 and 24:1 (Tonks et al., 2016). Tonks' study was in line with other studies showing increased concentration of Cer 18:0 (Coen et al., 2010, Amati et al., 2011). Cer 18:0 was also found to be increased in insulin-resistant rodent models (Turner et al., 2013). Kolak et al study showed Cer 24:1, to be increased in high fat diet (Kolak et al., 2007). Since adipose tissue acts as the main fat storage hub, lipid metabolism, and release, changes in the adipocyte lipidome may be reflected in the blood (Lapid

and Graff, 2017). The reason for this contradiction could be model related. Our model used primary human adipocytes, and the models used preclinically and clinically were mice and human adipose tissue or plasma respectively. The differences in the models might be the cause of the contradictory results observed here. Also, clinical studies used a large number of patients while we were limited in the availability of the adipocytes from donors who came under the selection criteria. A large number of samples could provide us with a clear picture about Cer. There are also other significant changes between ours and others studies: for example, the patients in other studies had been diabetic or obese for a long period of time while our data was taken from adipocytes exposed to AAPs 13 days post differentiation. This suggests that changes in lipids take a long time to be observed or detected. Although our data showed some changes, a longer study duration might produce different results. Another reason could be a compensatory increase in Cer brought about by the adipocyte to counter the decrease induced by AAPs; we undertook lipid profiling at the end of 13 days of adipocyte differentiation and therefore we have not been able to investigate the changes in Cer (and other lipid species) in between this timeline.

Interestingly, there are a few studies which indeed showed decreased levels of Cer. It has been proposed that Cer, along with other cellular functions, is also involved in adipocyte differentiation (Choi et al., 2011). Choi et al studied adipocyte differentiation in 3T3-L1 cells. Differentiation was induced by insulin and Cer analysis through HPLC was done after 6 days post differentiation. It was shown that Cer concentration in adipocytes decreased during adipogenesis compared to preadipocytes, while lipid droplets and TG content were increased, which is the characteristic feature of adipocytes (Choi et al., 2011). This data also coincided with

a study conducted on mice in which adipose tissue of *ob/ob* mice showed decreased levels of Cer compared to lean mice (Samad et al., 2006). TG synthesis in rat and human adipocytes require palmitic acid as a substrate. Palmitic acid is also required for SL biosynthesis so it was suggested that palmitic acid might be used for TG synthesis leading to a decrease in SL synthesis in adipocytes (Choi et al., 2011). Although our model was different from that of Choi et al, given AAPs accelerate adipogenesis as we have seen in our experiment (discussed in Chapter 3), increased diversion of palmitic acid to TG synthesis instead of SL during AAP-induced increase in adipogenesis could lead to decreased levels of Cer. This clearly shows the difficulties in dissecting insulin signalling pathways from adipogenesis in preclinical and clinical models and more research need to be done to explain the mechanisms involved.

Our study coincided with Blachnio-Zabielska et al data in which they showed a decreased content of Cer in the subcutaneous adipose tissue of obese non-diabetic and diabetic subjects. However, their methodology was different as they used adipose tissue instead of cultured adipocytes and measured total Cer content instead of individual species (Blachnio-Zabielska et al., 2012b). They also proposed that disease itself (in this case insulin resistance in conditions like obesity and diabetes) increased the metabolism of Cer. It was further proposed that the disease might activate CDases enzyme which breaks down the Cer, decreasing its levels. They found increased expression of CDases in their study (Blachnio-Zabielska et al., 2012a). They also showed in their study that Cer18 was increased in obese males while Cer 24 was increased in obese females. This gender dimorphism might be a significant factor in assessing the data. All the primary adipocytes used in our study were derived from

female donors; hence, according to Blachnio-Zabielska et al study, there could be a possibility that Cer 24, instead of Cer 18, was more involved in causing insulin resistance. This suggests that AAPs might increase Cer 24, while decreasing Cer 18 in female donors; this would however require more research to be undertaken in adipocytes established from both males and females.

To validate Cer data, we investigated the Cer metabolism pathway by analysing the gene expression of two important enzymes involved in Cer synthesis. Cer is formed by the coupling of palmitoyl-CoA and serine which produce ketosphinganine by the rate limiting enzyme SPTLC. There are 3 isoforms of SPTLC enzymes, SPTLC 1, 2, and 3 with SPTLC2 being the most abundant in white subcutaneous adipose tissue. Ketosphinganine is converted into sphinganine followed by the formation of dihydroceramide (DhCer) and then Cer by the enzyme DhCer desaturase. DhCer desaturase has two isoforms; DEGS1 is found in most tissues; however, mostly it is expressed in the liver, muscle and adipose tissue while DEGS2 is seen in the intestines (Chaurasia et al., 2016). Our data showed decreased expression of *SPTLC2* by CLO and OLA while ARI did not show any change. Blachnio-Zabielska and colleagues also studied Cer metabolising enzymes in subcutaneous adipose tissue in obese diabetic patients. Their study proposed that disease itself activates the CDases enzyme leading to a decrease in the content of Cer despite the increase in expression of *SPTLC* mRNA suggesting that there is more degradation of Cer than synthesis (Blachnio-Zabielska et al., 2012a). This has also been observed in a study conducted on *ob/ob* mice (Samad et al., 2006). However, our data showed a decrease in expression of *SPTLC 2* which suggests that AAPs might block the SPTLC2 enzyme directly leading to a blockade of Cer synthesis or AAPs might activate CDases, although we did not measure the

effect of AAPs on CDases in our data, leading to decreased levels of Cer species. However, ARI showed no change in expression which suggests normal synthesis of Cer in the presence of ARI, showing ARI to have the least metabolic toxic effect compared to CLO and OLA.

The final reaction involved in *de novo* Cer synthesis is mediated through enzyme DEGS1. It has been documented that the decreased expression or downregulation of DEGS1 increases the DhCer/Cer ratio in cellular models (Barbarroja et al., 2015). DhCer is also considered a modulator of cell cycle, apoptosis and autophagy. Genome-wide association studies in mice identified *DEGS1* as a candidate gene associated with lipid accumulation (Barbarroja et al., 2015). Understanding DEGS1 and its associated Cer metabolic pathway not only provides relevance but could also be considered as a potential therapeutic target for obesity-associated insulin resistance. Barbarroja et al showed that *DEGS1* gene expression was found to be decreased in a high-fat diet mouse model and also in genetically induced obese mice (*ob/ob*). Our data coincided with this study in terms of the effect of CLO and OLA on *DEGS1* mRNA expression. ARI however, showed an opposite effect with a trend to increase, but the result was statistically non-significant. Although our model was based on primary human adipocytes compared to Barbarroja's model (mice adipocytes), it suggests that reduction in *DEGS1* might be one of the potential mechanisms by which AAPs act and decrease Cer synthesis. Barbarooja et al also showed that this downregulation only happened in adipose tissue while liver and skeletal muscle did not show any change, demonstrating its specificity to adipose tissue (Barbarroja et al., 2015). Downregulation was also shown in visceral AT of obese patients showing an association of DEGS1 with metabolic diseases such as obesity or T2DM. As obesity

is characterised by low-grade inflammation, it has been documented that the modulators of obesity-associated inflammation cause down-regulation of DEGS1 compared to normal individuals (Barbarroja et al., 2015). This suggests that AAP might cause inflammation resulting in decreased *DEGS1* leading to decreased levels of Cer resulting in insulin resistance.

Barbarroja and colleagues also studied the association between DEGS1 and adipogenic transcription markers. In an *in vitro* study on 3T3-L1 adipocytes, a direct correlation of pparγ2 and *degs1* was shown. *In vivo* validation conducted on adipose tissue of PPARγ2KO (PPARγ2 knock-out mice) and POKO mice (a crossed *ob/ob* mice, having capability of storing large amounts of fat, with mice deficient of PPARγ to generate 'POKO mice', having increased circulating levels of glucose and fat) showed decreased mRNA expression of *DEGS1* in PPARγ2KO and POKO mice (Medina-Gomez et al., 2007). Decreased expression of PPARγ2 might lead to an alteration in adipocyte differentiation so DEGS1 indirectly affects adipocyte differentiation. However this contradict with our previous data where it showed increased PPARγ expression (chapter 3 & 4) while DEGS1 data showed decreased expression by AAPs (chapter 5). This contradiction might be due to (i) change in methodology as we performed our experiment on primary human adipocytes while Medina-Gomez study was done on mice (ii) Our data was performed on 3-5 donors which might not reflect the effect, more donors are required to see effect of AAPs on PPARγ and DEGS1 and their interaction. It has also been shown that downregulation of DEGS1 expression in DEGS1 knock down (KD) 3T3-L1 cells was associated with down-regulation of SPTLC and Cer synthase (*Cer6*) enzymes, suggestive of physiological readjustment of the biosynthetic pathway to compensate for the

dysregulation of DEGS1. It can be postulated that AAPs like CLO and OLA, but not ARI, decrease *DEGS1* expression leading to alteration in the DhCer/Cer ratio resulting in an increased content of DhCer. This might suggest a potential role for DhCer in insulin resistance. There is a possibility that AAPs result in increase in DhCer levels when it results in a decrease in Cer as shown in our data (Mamtani et al., 2014, Weir et al., 2013).

In conclusion, our lipidomic study suggests that metabotoxic AAPs, particularly at higher concentrations, might act directly on adipocytes and cause global changes in the adipocyte lipidome. AAPs such as CLO and OLA resulted in a reduction of several Cer species and this may be modulated by their interaction with various enzymes that mediate the Cer synthesis pathway. However, our data contradicted with some of the published data; this could be because of differences in methodology, duration of studies, gender dimorphism and individual lipid profiles investigated. It should be noted that there is also widespread contradiction between various studies on Cer in the existing literature. Detailed research with the use of modern technology is now required to find the mechanistic basis of various lipid species identified by our study to investigate their role in AAP-induced metabolic toxicity; this will be important not only for mechanism elucidation but also for the development of biomarkers and therapeutic strategies to reverse/arrest AAP-induced metabolic toxicity.

Chapter 6

Final Discussion

6.1 Overview of the thesis

Schizophrenia is a chronic psychiatric disorder affecting 0.5-1% of the world's population. It is characterised by positive symptoms such as hallucinations and delusions; negative symptoms such as lack of motivation, social withdrawal, disorganised speech and behaviour; and, cognitive symptoms. These symptoms typically appear in adolescence and early adulthood. It has been estimated that people suffering from schizophrenia have a 20% shorter life expectancy than the general population, with cardiovascular disease being the leading cause of death (Heald et al., 2017). Disturbance in connections and changes in neurotransmitters such as dopamine, glutamate, serotonin and acetylcholine in various brain regions including the midbrain, nucleus accumbens, thalamus and prefrontal cortices have been proposed as being involved in the pathophysiology of schizophrenia (Lewis and Lieberman, 2000). AAPs are considered as the primary drugs to treat schizophrenia because of their better tolerability, improvement in schizophrenic symptoms and better adverse effect profile as compared to typical antipsychotics which cause extrapyramidal adverse effects. However, long term use of AAPs is associated with metabolic syndrome which consist of obesity, glucose intolerance, insulin resistance, T2DM, dyslipidemia and hypertension, all leading to cardiovascular disease thereby contributing to overall mortality and morbidity in schizophrenia (Sapra et al., 2018). The prevalence of metabolic syndrome is considered to be 23-50% in AAP-treated schizophrenic patients (Gautam and Meena, 2011, Bai et al., 2011). Among AAPs, CLO and OLA are considered to be associated with greater risk; QTP, RISP, ILP and PLP intermediate risk; while ARI, AMI and ZIP are associated with smaller risk of adverse metabolic effects (Bak et al., 2014). It is of utmost importance to find the mechanisms by which AAPs cause metabolic adverse effects so that the benefit-risk profile of these drugs

can be improved. Various mechanisms have been proposed which include lipid accumulation, dyslipidemia, inflammation, insulin resistance and mitochondrial dysfunction; however, no absolute mechanism has emerged (Coccurello and Moles, 2010, Masaki et al., 2004). White adipose tissue has been a subject of interest for several years in metabolic disease research and is now considered a 'hot spot' in biomedical research (Trayhurn and Wood, 2005). Metabolic syndrome is associated with dysregulated adipose function and metabolism affecting not only its fat storage activity but also its endocrine functions. The understanding of adipose tissue biology and the deleterious changes associated with obesity could potentially throw light on the mechanisms involved in metabolic toxicity caused by AAPs (Gustafson et al., 2007). Adipose tissue secretes various products such as adiponectin, leptin, resistin, TNF-α, MCP-1, retinol binding protein 4, and adipocyte-type fatty acid binding protein, some of which elicit their effects on peripheral tissues and thereby play a role in glucose and lipid homeostasis. Transcription factors especially PPARγ, CEBPα and SREBP1 also play a role in maintenance and maturation of adipocytes. Disturbance in homeostasis of these transcription factors by AAPs might also lead to metabolic toxicity (Oda, 2008).

The main aim of this PhD project was to characterise the effect of AAPs on adipocyte function and metabolism in order to improve our understanding of the molecular mechanisms that are involved in AAP-induced metabolic toxicity. For this we used two different *in vitro* models: a murine adipocyte cell line, 3T3-F442A, and primary human subcutaneous adipocytes. We preferred the 3T3-F442A preadipocyte model as it showed more advanced commitment for differentiation compared to the 3T3-L1 or OP9 model (Ruiz-Ojeda et al., 2016). Chapter 2 focused on the effects of CLO and

OLA as exemplar AAPs with high metabolic toxic profile, and ARI as a drug with very low metabolic liability. We characterised the effect of the above AAPs on adipocyte lipid accumulation, adipokine secretion and the expression of various adipogenic transcription factors using a chronic drug toxicity model. AAPs were used in two different concentrations: a therapeutically relevant concentration, and a higher concentration which reflect potential accumulation of these drugs in the adipose tissue. This would therefore resemble a clinical scenario where patients have been exposed to AAPs for a long period of time. Our lipid accumulation data coincided with other *in vitro* (Hu et al., 2010, Tsubai et al., 2017) as well as clinical studies (Francesco and Cervone, 2014) which suggested that CLO and OLA but not ARI increased adipogenesis by acting directly on adipocytes leading to lipid accumulation. However this also contradicted with some other studies which showed no effect of AAPs on fat cell formation (Hauner et al., 2003). The contradiction may have arised due to a difference in methodology; or models used; or drug treatment duration. Our data also suggested the interaction between AAPs and adipogenic transcription factors as an underlying mechanism for AAP-induced metabolic toxicity. PPARγ, considered as a master regulator of adipogenesis, without which adipocytes cannot mature and perform physiological action was one of them. PPARγ is considered to be involved in glucose and lipid homeostasis thus improving insulin resistance (Liu et al., 2014). Our data coincided with other studies, showing increased PPARγ expression following incubation of adipocytes with CLO while OLA and ARI did not show any change (Sarvari et al., 2014, Sertie et al., 2011). This suggests that AAPs, especially CLO, might activate PPARγ thereby resulting in increased adipogenesis, leading to increased lipid accumulation and weight gain. Lipin 1, another transcription factor on which the effect of AAPs was reported for the first time, was considered to act as a

key factor for the maintenance and maturation of adipocytes. Our data showed increased lipin 1 expression with CLO but not OLA or ARI. PPARγ and lipin1 are also interrelated as lipin1 co-activates PPARγ2 and then regulates the network between PPARγ2 and C/EBPα. This suggests that AAPs might activate PPARγ indirectly through lipin1 which leads to increased adipogenesis and lipid accumulation; however, more research is required to see the effect of AAPs on lipin1 (Koh et al., 2008).

Insulin has an inhibitory effect on FFA release but, due to insulin resistance, this is inhibited and more FFA will be released, either stored in adipose tissue or peripherally leading to tissue steatosis. FFA also causes glucose intolerance by decreasing glucose intake in muscles leading to diabetes (Adiels et al., 2005). Our data coincided with previous studies which showed increased FFA with CLO and OLA except ARI, which did not show any change (Kaddurah-Daouk et al., 2007, Canfran-Duque et al., 2013, Jassim et al., 2012). This suggests that increased FFA levels might be one of the mechanisms by which AAPs cause metabolic adverse effects leading to insulin resistance. Our study on 3T3-F442A also investigated the effect of AAPs on adipokines. Adiponectin is considered as an insulin sensitiser and anti-inflammatory factor released from adipocytes and activates AMPK which increased the fatty acid oxidation. This step kick-start a series of reactions leading to increased uptake of glucose in muscle cells where it is converted into lactate. This diversion of glucose to muscle resulted in a reduction in liver gluconeogenesis triggering more uptake of glucose from plasma. Increased fatty acid oxidation, decreased gluconeogenesis in the liver and decreased glucose levels in plasma ultimately improves peripheral insulin sensitivity. However, decreased adiponectin levels lead to insulin resistance and

diabetes (Gil-Campos et al., 2004). It has been shown by various clinical studies (Bartoli et al., 2015b, Shah et al., 2008a) and an *in vitro* study on 3T3-L1 (Tsubai et al., 2017) that AAPs reduce adiponectin secretion. However, we observed contradictory data in our experiment with CLO where it showed an increased adiponectin secretion when treated with CLO while OLA and ARI showed decreased secretion. We also observed a discrepancy in the level of proinflammatory markers such as IL-6 and TNF-α secreted by the drug-treated adipocyte. Inflammation is considered one of the mechanisms by which AAPs cause metabolic toxicity. It has been postulated that pro-inflammatory markers like IL-6 and TNF-α act on insulin receptor and block its phosphorylation which leads to insulin resistance (Makki et al., 2013). Various clinical studies (Pollmacher et al., 2000, Makki et al., 2013) showed increase in IL-6 and TNF-α levels after giving AAPs while our IL-6 data showed no change with all the drugs while TNF-α was only increased with CLO (Makki et al., 2013). We can argue here that our data spanned only 10 days with only 4 drug additions while, clinically, patients take AAPs for long period of time which could be the cause of the contradictory data.

We then used a primary human subcutaneous model to validate the murine adipocyte results and also to explain some of the discrepant data we obtained in the murine model.

Preadipocytes (Chapter 3) obtained from healthy female abdominal subcutaneous adipose tissues were subjected to the same chronic AAPs treatment as preformed with the murine adipocytes. Interestingly, the human adiponectin data coincided with what was observed clinically; we observed a clear decrease in adiponectin secretion with

higher doses CLO and OLA which was not observed with ARI. Importantly, the reduction on adiponectin with therapeutic concentrations of CLO and OLA were minimal. The CLO accumulation assay which was performed in both primary human adipocytes and murine adipocytes showed that primary human adipocytes accumulated 20 times more CLO than murine adipocytes. This may suggest that the uptake of CLO into murine adipocytes was very less. This could be the reason for the contradictory result we observed in our murine model. This may also suggests that the uptake of AAPs in murine cells is operated through a different mechanism compared to primary human adipocytes. We have used the primary human adipocytes as our preferred model in subsequent experiments.

Our data showed that CLO and, to some extent, OLA cause increased lipid accumulation in primary human adipocytes whereas ARI showed no change. This coincided with other studies performed on isolated subcutaneous adipose tissue (Hemmrich et al., 2006, Himmerich et al., 2011, Pavan et al., 2010). This suggests that AAPs directly affect the adipocytes and increase lipid accumulation leading to obesity and weight gain. Our *PPARγ* mRNA data did not show any change after using AAPs which coincided with other studies performed on primary human adipocytes (Nimura et al., 2015, Sarvari et al., 2014, Sertie et al., 2011). Our PPARγ protein data did show a trend to increase with CLO and OLA but not ARI; however, we also observed a high degree of variability in response between our biological replicates (adipocyte donors) and, therefore, these results were statistically non-significant. Due to limitations in the availability of adipocytes from donors plus the difficulty of performing a chronic toxicity experiment in a large number of replicates, we were restricted to only 3 biological replicates. Increased sample size could reduce this variability. However, when these samples were analysed individually, the trend of expression was consistent

between all 3 biological replicates. Lipin1 was investigated for the first time; however, the result varied between biological replicates for the individual doses of AAPs and it was difficult to conclude how lipin1 was affected by AAPs. As mentioned earlier, lipin 1 is important for the maturation and maintenance of adipocytes, therefore future studies with an increased sample size are required to study its role in metabolic toxicity. Decreased levels of adiponectin have been observed in insulin resistant patients and in conditions such as obesity and diabetes mellitus (Jin et al., 2008). Our data coincided with clinical studies where CLO and OLA but not ARI showed decreased adiponectin secretion (Klemettila et al., 2014, Pollmacher et al., 2000) which suggests that AAPs act directly on adipocytes and decrease adiponectin secretion which may lead to insulin resistance. Another important adipokine is leptin, the role of which is to suppress food intake and cause weight loss; our data showed an increase in leptin levels with CLO but not OLA while ARI showed a decrease in leptin levels which coincided with previous studies (Potvin et al., 2015, Paredes et al., 2014, Jin et al., 2008, Ak et al., 2013, Tanaka et al., 2008). Increased leptin levels without weight loss in obese patients have been observed previously which suggests the development of leptin resistance in these patients (Potvin et al., 2015). This suggests that AAPs could cause leptin resistance leading to increase in leptin levels. Inflammation is also considered one of the important mechanisms by which AAPs cause metabolic toxicity. The pro-inflammatory markers act on insulin signalling mediators such as IRS1 and block its phosphorylation leading to insulin resistance (Makki et al., 2013). Our IL-6 data coincided with previous studies where CLO and OLA but not ARI showed increased IL-6 levels. This suggests IL-6 as one of the main inflammatory markers which plays a role in metabolic toxicity by potential blockade of insulin signalling leading to insulin resistance.

Minimising weight gain and metabolic adverse effects caused by AAPs in schizophrenic patients is of clinical and societal importance (Sugawara et al., 2017). Chapter 4 focused on adjunctive therapeutic strategies to investigate whether they can reverse or arrest the deleterious effects of AAPs on adipocytes. Previous studies have shown co-administration of various drugs such as TOPI, sibutramine, MET, nizatidine, amantadine, ARI, and, modafinil with AAPs to improve the metabolic toxicity profile. Various RCTs showed improvement in metabolic adverse effects after concomitant use of MET, ARI and TOPI (Zimbron et al., 2016). However, previous studies could not recommend any specific drug for broad clinical usage due to limitations in clinical studies; moreover, none of these studies have investigated the mechanisms behind therapeutic reversal of AAP-induced metabolic toxicity (Maayan et al., 2010). We used MET, ARI, TEL as potential therapeutic options and coincubated with CLO in the primary human adipocyte model. We selected CLO as it showed prominent changes in adiponectin secretion and PPARγ expression compared to OLA, as described in Chapter 3. MET, drug of choice in T2DM, was reported to cause weight loss and improved glucose levels and insulin sensitivity in diabetic subjects (Baptista et al., 2008, Baptista et al., 2007). TEL is an angiotensin II receptor blocker and mainly used as a antihypertensive. It was reported that TEL showed improvement in insulin resistance in metabolic syndrome patients (Derosa et al., 2007). Preclinical and *in vitro* studies have also proposed the role of TEL in improving insulin resistance through PPARγ activation (Benndorf et al., 2006, Souza-Mello et al., 2010, Foryst-Ludwig et al., 2010, Pushpakom et al., 2017). Makita's study showed improved adiponectin levels in glucose intolerant patients showing the efficacy of TEL in insulin resistant patients (Makita et al., 2008). ROSI, used as a positive control,

which has PPARγ agonistic activity, also showed improvement in insulin sensitivity; however, it was withdrawn clinically due to cardiovascular complications (Singh et al., 2007). Our data showed that all adjunctive drugs cause a trend to reverse the CLO-mediated reduction in adiponectin secretion although the result was statistically non-significant due to the high inter-individual variability observed. Our studies coincided with other studies showing improvement in adiponectin secretion on giving these adjunctive drugs alone to metabolically deranged patients with conditions like obesity, diabetes mellitus or insulin resistant (Wang et al., 2013, Zulian et al., 2011, Makita et al., 2008). This suggests that adjunctive drugs may act directly on adipocytes and maintain adiponectin levels leading to improved insulin sensitivity. Our PPARγ data showed that all adjunctive drugs except 10μM Tel reversed PPARγ expression when given along with a therapeutic dose of CLO. This suggests that adiponectin and PPARγ are interrelated and adjunctive drugs might activate PPARγ which then activates adiponectin leading to an improvement in insulin resistance. This further suggests that adjunctive drugs not only maintain adipogenesis, as PPARγ is a master regulator of adipogenesis, but also improve insulin resistance through adiponectin. Data of adjunctive drugs with high dose CLO showed no change in expression by ARI, MET and TEL5μM. This suggests that the concentration we used for adjunctive drugs may not be sufficient to counter the effects of high dose of CLO (20μM). However, 10μM TEL showed decreased PPARγ expression when given both with therapeutic and high dose of CLO despite increased adiponectin secretion. This might be due to TEL's partial agonistic activity which showed that increase dose of TEL (10μM TEL) have more antagonistic effect instead of agonistic effect leading to decreased PPARγ expression. Our adjunctive drugs data with CLO suggest that these drugs can reverse the AAP-induced metabolic adverse effects through adiponectin or PPARγ or

combination of both. Various studies also suggested a positive correlation between PPARγ with adiponectin and reported that PPARγ agonist stimulates serum adiponectin secretion which was confirmed from our data (Guan et al., 2002, Combs et al., 2002, Yang et al., 2004).

Chapter 5 focused on how AAPs may affect the adipocyte lipidome on a global using cultured primary human adipocytes. Lipidomics profiling of adipocytes is a new field; it has the potential to uncover novel mechanisms of pathogenesis and identify new biomarkers of toxicity. Lipogenesis in adipocytes involves numerous complex pathways, regulated by various transcriptional factors especially PPARγ, CEBPα and SREBP1.These transcriptional factors induce various other genes which are involved in fatty acid and glucose uptake. Various enzymes and hormones, the most important of which is insulin, also regulate these pathways. Numerous studies have reported increased levels of DAG, CE, TAG and Cer in the plasma of obese and diabetic patients as compared to normal subjects (Tonks et al., 2016, Rauschert et al., 2016). Our PCA and PLS-DA data showed that higher dose of CLO and OLA but not therapeutic concentrations cause profound change in global lipid profile while ARI did not show much change in the adipocyte lipidome. This change in global lipid profile by CLO and OLA clearly suggest that these AAPs when used chronically could accumulate in adipose tissue leading to deleterious effects on the adipocyte lipid content and contribute to AAP-induced cardiometabolic toxicity. Our results coincided with Daouk et al's study which also showed global changes in lipids after giving OLA but not ARI in schizophrenic patients suggesting a role of adipocyte lipids in causing metabolic toxicity. Previous studies on obese and diabetic patients have shown remarkable changes in GLP and SL compared to other lipids (Alshehry et al.,

2016). Among SL, Cer showed an association with CVD (Spijkers et al., 2011). Cer is synthesised de novo by a combination of serine and palmitoyle CoA regulated by enzymes SPT. Cer regulate cellular function including proliferation, differentiation, growth arrest and apoptosis. On accumulation in tissues during obesity, it acts as a toxic lipid leading to dysregulation of cellular functions resulting in insulin resistance (Hage Hassan et al., 2014). It has been proposed that Cer blocks the IRS-1 and PI3K insulin signalling pathway thus blocking insulin stimulated glucose transport and glycogen synthesis leading to insulin resistance (Summers et al., 1998, Schmitz-Peiffer et al., 1999). High levels of Cer also lead to increased ROS which causes mitochondrial dysfunction leading to apoptosis of cells. This will form lipid metabolites that block the insulin signalling pathway (Hage Hassan et al., 2014). Preclinical (Samad et al., 2006) as well as clinical studies on insulin resistant patients (Amati et al., 2011, Gertow et al., 2014) showed increased levels of Cer suggesting its association with metabolic deranged conditions like obesity and T2DM. Cer could be a potential lipid biomarker by which AAPs act on and produce metabolic toxicity. We measured Cer 18 species levels as it has been suggested that it is associated with insulin resistance and its levels have been found to be increased in adipose tissue in obese patients (Kolak et al., 2007, Turpin et al., 2014). Contrary to the published data in obese patients (Tonks et al., 2016, Coen et al., 2010) and an insulin resistant rodent model (Turner et al., 2013) where increased Cer 18 levels were shown, our data with CLO and OLA but not ARI showed decreased Cer 18 species levels (16:0, 18:0, 18:1, 20:0, 22:0, 22:1, 24:0 ,24:1, 26:0, 26:1). The reason for this contradiction could be (i) the difference in the model as previous studies were on mice or human adipose tissue or plasma compared to ours which used cultured human adipocytes; (ii) the difference in the number of samples as we used only five donors due to a limitation in the

availability of donors compared to the large number of patients in previous studies (a large number of samples could lead to different data); (iii) the duration of the study as obese or diabetic patients were in this condition for a long period of time while our study span was about 13 days. Long study duration with more drug additions might produce the same results as mentioned for previous studies. However, there are a few studies which showed decreased levels of Cer in subcutaneous adipose tissue of obese and diabetic subjects arguing that the disease (insulin resistance in obesity and diabetes) itself increased the metabolism of Cer (Blachnio-Zabielska et al., 2012b, Blachnio-Zabielska et al., 2012a). Blachnio-Zabielska and colleagues' study reported that obese males showed an increased content of Cer 18 while obese females showed Cer 24 respectively. This may suggest the possibility that Cer 24 instead of Cer 18 was more involved in causing insulin resistance. As we used primary human adipocytes from female donors in our study there could be possibility that AAPs might increase Cer 24 while decreasing Cer 18 however more research is required involving both male and females donors to find the changes in Cer levels. Although we did not measure Cer 24 in our study, a decrease in Cer 18 might be reflected in our data because of this gender difference. Another study by Choi and colleagues on 3T3-L1 showed decreased Cer content in adipocytes compared to preadipocytes as adipogenesis progressed. They proposed that TG synthesis requires palmitic acid, which is also required for synthesis of Cer, so in a condition of increased lipid droplet formation, palmitic acid may be used for synthesis of TG instead of Cer leading to decreased Cer content (Choi et al., 2011). As our AAP data showed more lipid accumulation, as discussed in Chapter 3, there is a possibility that palmitic acid was used for synthesis of TG which resulted in decreased levels of Cer. However, more detailed research is required to explore this. We then validated our Cer data by

investigating the Cer synthesis pathway through quantitative gene expression analysis of important Cer synthesis enzymes, SPTLC2 and DEGS1. Cer is formed by coupling palmitoyle CoA and serine by rate limiting enzyme SPTLC2 while DEGS1 convert DhCer to Cer. Our data showed decreased mRNA expression of *SPTLC2* and *DEGS1* with CLO and OLA but not ARI. This suggests that CLO and OLA might block these enzymes leading to decreased Cer content. Our DEGS1 data also coincided with Barbarroja's study in which decreased *DEGS1* mRNA expression was shown in isolated adipocytes from a high fat diet mouse and genetically induced mice *(ob/ob*) (Barbarroja et al., 2015) leading to an increased DhCer/Cer ratio, increased DhCer levels and decreased Cer levels. This downregulation of DEGS1 was also observed in the visceral AT of obese patients suggesting an association between DEGS1 and DhCer levels with metabolic disease (Barbarroja et al., 2015). DhCer could have a potential role in causing insulin resistance in metabolic deranged patients. Although we did not measure DhCer levels in our experiment, there is a possibility that AAPs block DEGS1 enzymes leading to increased DhCer levels, while decreasing Cer levels. This increase in DhCer levels might cause insulin resistance leading to cardiovascular complications. More research is required to explore DhCer as recent reports have shown a positive correlation between DhCer and insulin resistance in obese subjects rather than Cer (Weir et al., 2013, Mamtani et al., 2014).

6.2 Conclusion

In conclusion, novel data has been presented in this thesis that could add to the evidence base on the pathogenesis of metabolic toxicity caused by AAPs. These data relate to increased adipogenesis, the role of transcription factors, inflammation, changes in adipokines and lipids levels. However, the results clearly showed that the

effect of metabotoxic AAPs (CLO and OLA) on adipocytes (lipid accumulation, adipokine release, adipogenic transcription factor expression and lipid content) significantly varies from that of a metabolically friendly AAP (ARI). This thesis also highlights the importance of (i) adipocyte lipidomics and its role in insulin resistance and, (ii) the potential of adjunctive therapeutic strategies in reversing AAP-induced metabolic adverse effects. This will help to develop new potential targets, biomarkers or drugs to treat AAP-induced metabolic toxicity. For this purpose, a greater understanding of the mutual interaction between drugs and peripheral tissues such as liver, skeletal muscle and adipose tissue that mediate insulin resistance is required.

6.3 Future work

Various studies have postulated different mechanisms for AAP-induced metabolic toxicity but the absolute mechanisms are unclear. There is still a lot of work which needs to be done in this area which is discussed below:

1-Various animal studies have been conducted to find the underlying mechanism of AAP-induced metabolic toxicity but still no absolute model has been presented which mimics schizophrenic-like conditions. Studies on animal models also showed a lot of variation in data among species. An animal model with knock out models of adipogenic genes such as PPARγ and lipin1 may be helpful to understand the mechanism involved in AAP-induced metabolic toxicity.

2- Our 3T3-F442A preadipocyte cell line model showed a decreased accumulation of CLO in the cells. It is necessary to perform detailed studies on adipocyte transporters to find the uptake of various drugs into the adipose tissue.

3- There is very little information about the effect of AAPs on the lipid profiles of mature adipocytes and this need to be investigated using advanced technologies such as UPLC/MS. This would provide a large set of data to help find more comprehensive conclusions in terms of finding potential biomarkers, drug targets and better treatment options.

4- A large cohort (donors) of cultured primary human adipocytes is required to study the effect of AAPs on metabolic parameters. Factors such as age, sex, drug dosage, drug pharmacokinetics and methodology should be carefully selected which might represent the data in a different way.

5- A few studies have been performed on adjunctive therapy given concomitantly with AAPs; however, these small studies showed promising result in reversing metabolic toxic effects. A detailed study is required to explore the effect of various adjunctive strategies to identify the best candidate drugs that can be taken forward into in vivo studies and then on to clinical trials.

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